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The first year of this contract ~~has been~~ devoted to the conjugation of selected antivirals (ribavirin) and immunomodulators (MDP) to neoglycoprotein carriers (poly-L-Lysine or BSA). ~~Our~~ in vivo virus challenge studies suggest that MDP-BSA and Poly-L-Lysine conjugates are more effective than free MDP in enhancing resistance to HSV-1 hepatitis and pneumonitis. Moreover, conjugated MDP was more effective in enhancing RES function than was free drug.

Squirrel monkeys ~~have been~~ shown to be useful in the evaluation of orally administered immunomodulating agents which have been ~~shown to be~~ effective in mice. Studies on a dengue/squirrel monkey model have been initiated and it is anticipated that it should soon be ready for the evaluation of both liposome-encapsulated or conjugated antivirals and immunostimulants. This primate model will be useful in establishing the preclinical efficacy of promising new drugs.


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19. ABSTRACT (continued)

A human macrophage antiviral assay ~~has been shown~~ to be a valuable tool for *in vitro* evaluation of targeted antivirals and immunostimulants. This assay system has been used to study both RNA and DNA viruses and has allowed us to examine the biological activity of free and conjugated drugs. For example, ~~we have shown that~~ conjugation of the nucleoside analogue, PMEA, to poly-L-Lysine could be accomplished without any loss of biological activity as determined by the *in vitro* HSV-1/macrophage assay. Ongoing studies continue to address the *in vivo* efficacy of conjugated versus liposome-encapsulated antivirals and immunostimulants.

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Eugene P. Mayer Eugene P. Mayer 5-24-89
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Abdul Ghaffar 5/24/89

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# I. INTRODUCTION

## A. BACKGROUND AND HYPOTHESIS

A number of clinically proven antivirals, such as amantadine, iododeoxyuridine, adenine arabinoside, acycloguanosine, ribavirin, and azidodeoxythymidine are currently in use. In addition, several potentially useful antivirals (e.g. S-HMPA, selenazole, WIN 5177, arildone, phosphonoformic acid) are currently under investigation. There are, however, toxicity problems associated with the use of antivirals at doses required to provide sustained drug levels in virus infected organs. Because of the many similarities in synthetic "machinery" and pathways used by both viruses and mammalian cells, these toxicity problems may be difficult to overcome. Furthermore, in some virus infections even the presence of a highly active antiviral in the infected target organ may not be sufficient to prevent or limit disease. Thus, a delivery system which can provide slow release of an antiviral, alone or in combination with other effector substances (e.g., immunostimulants, antibody, interferon), at the site of primary virus replication may be highly desirable. We believe, that with the recent technological advances in the development of carrier vehicles designed to protect nontarget environments from the drugs they carry, and facilitate slow release of one or more drugs at sites at which they are needed, many of the problems currently associated with antiviral chemotherapy can be minimized.

### A.1 Rationale and Theoretical Considerations for the Use of Targeted Drug Carriers

Successful drug use in medicine is often jeopardized by the failure of drugs that are otherwise active *in vitro* to act as efficiently *in vivo*. This is because in the living animal drugs must, as a rule, bypass or traverse organs, membranes, cells and molecules that stand between the site of administration and the site of action. In addition, drugs may be prematurely excreted or inactivated; thus, an effective therapeutic drug concentration may not be obtained at the site of infection. While this problem can be overcome by increasing dosage, this often results in toxicity. For example, a number of antiviral agents (ribavirin, adenine arabinoside, phosphonoformic acid, azidodeoxythymidine) have been developed which are highly effective *in vitro* in preventing virus replication and/or cell death; however, their systemic use in man is limited by the induction of toxic effects which occur at dosages required to maintain effective drug concentrations in the infected organ. In particular, sustained treatment often results in leukopenia and subsequent immunosuppression (1,2) which may affect the outcome of treatment since recovery from most viral infections involves the cooperation of host immune responses. There is now

growing optimism that these problems may be resolved or minimized with the use of carrier vehicles such as liposomes, monoclonal antibodies or conjugated proteins. These vehicles not only protect the immune system from the drugs they carry but also deliver and facilitate drug release at site(s) at which they are needed.

A prerequisite for the successful use of antiviral carriers is that they recognize the target site and release drug in an active form. Thus, the antiviral must be linked to a carrier in a stable fashion so that it remains attached throughout its journey from the site of inoculation to the target. For this to occur, the drug-carrier linkage must be resistant to the various enzymatic and physiochemical conditions prevailing in the bloodstream and extracellular fluids. This stability also implies that the drug remains inert as long as it is associated with its carrier so that it will be inactive prior to reaching the target area. A corollary of this requirement is that there be a mechanism by which the drug will be activated after reaching its destination. Three possibilities exist for the activation of carrier bound drugs: 1) endocytosis and release in acidic endosomes; 2) extracellular activation at cell surfaces by plasma membrane associated enzymes; and 3) intracytoplasmic penetration via transmembrane passage.

During the past decade, the use of carriers for the selective targeting of antitumor drugs has been advocated with increasing frequency. This has led to numerous reports on the association of drugs or toxins such as anthracyclines, methotrexate, bleomycin, chlorambicin, cytosine arabinoside (ara-C), and ricin with carriers such as DNA (3,4), liposomes (5,6), monoclonal antibodies (7-9), hormones (10,11), red blood cell ghosts (12), neoglycoproteins (13) and other proteins (14,15). Most of these carriers have the ability to selectively interact with target cell surfaces and are subsequently endocytosed and transferred to the lysosomal compartment where free drug is released when the bond between drug and carrier is hydrolyzed by lysosomal enzymes (16). In contrast, some liposomes deliver their contents directly into the cytoplasm following fusion with the target cell membrane (17). This mode of delivery is useful for antivirals which are susceptible to lysosomal enzymes since membrane fusion is a mechanism through which drug contact with lysosomes can be bypassed. In addition, liposomes are useful in the delivery of poorly soluble agents such as arsenicals in the treatment of leishmaniasis (18) and lipophilic drugs such as MTP-PE in the treatment of herpesvirus infections (19,20). Moreover, they provide a means to deliver drugs that cannot be transported across cell membranes.

### A.1.1 Liposomes

Four basic liposome designs: 1) multilamellar; 2) small unilamellar; 3) large unilamellar; and 4) vesicles produced by reverse phase evaporation, have been used as carrier systems for

enhancing the pharmacological activity of drugs and the incorporation of macromolecules into cells. The most commonly used methods for the preparation of liposomes are the multilamellar vesicles originally described by Bangham et al. (21), and the small unilamellar vesicles initially described by Papahadjopoulos and Miller (22). The main drawbacks of these "classical" liposome preparations are the wide heterogeneity in size distribution and number of lamellae, the relatively low trapping efficiency in the aqueous space and/or their inability to encapsulate large macromolecules due to their small internal volume (23). In contrast, newer methods in the preparation of large unilamellar and reverse phase evaporation liposomes have resulted in carriers with larger internal volumes and better encapsulation properties. For example, a reverse evaporation process has been used to produce unilamellar vesicles capable of encapsulating substantial amounts of ara-C (24). These liposomes were composed of phophatidylserine and cholesterol and were stable in serum. Furthermore, their distribution to lung, liver and spleen following intravenous inoculation could be controlled, in part, by altering one or more physical features (e.g., size, membrane fluidity and charge,) or by embedding of glycoconjugates, proteins etc.

Comparison of pulmonary retention of liposomes of differing size, surface charge, and composition following iv injection revealed that large multilamellar or reverse phase evaporated liposomes arrested in the lung more efficiently than small unilamellar liposomes of identical lipid composition. In addition, multilamellar and reverse phase evaporation vesicles containing negatively charged amphiphiles arrested in the lung more efficiently than neutral or positively charged structures which accumulated in the liver and spleen (25). Reverse phase evaporation carriers were superior to multilamellar carriers with respect to their loading capacity, however, the latter provided a more prolonged drug release.

Liposomes containing glycolipid have a preferential affinity for hepatocytes due to a galactose binding membrane lectin (26). Using liposomes which varied in size and galactosyl lipid content (galactocerebroside), Rahman et al. were able to show more selective targeting of small unilamellar vesicles to hepatocytes (27). Similarly, large liposomes containing galactocerebroside produced a substantial enhancement of liposome-encapsulated primaquine activity against the schizont stage of malarial parasite which is known to reside in hepatocytes (18).

In many instances, liposomes are avidly taken up by tissue associated macrophages which are present in most organs. Selective uptake of these vehicles by macrophages can be quite useful and can be exploited in some infectious diseases; thus, Alving et al. (18) used liposome-encapsulated antimonial drugs to treat leishmaniasis, a parasitic infection in which the macrophage is the primary target

of infection. Encapsulated drug preferentially accumulated in these cells and killed the parasite with minimal toxicity for nontarget cells.

In addition to the examples cited above, liposomes have been used as carriers to enhance the immunogenicity of a variety of viral proteins including hepatitis B surface antigen, vesicular stomatitis virus glycoproteins, and adenovirus capsid proteins. Encapsulation of these proteins and inoculation into animals resulted in an increased production of serum neutralizing antibodies (28-30). In at least one study, in which purified major surface glycoproteins of VSV were encapsulated into liposomes, both specific humoral and cell mediated immunity were enhanced (28).

Recently, attempts to load antivirals into freeze-dried liposomes has resulted in improved encapsulation with minimal drug-leakage upon storage (personal communication, Dr. Peter van Hoogevest). This is particularly significant since it should now be possible to more easily load a variety of antivirals and/or immunostimulants into this type of carrier. We have recently encapsulated both ribavirin and muramyl tripeptide phosphatidylethanolamine (MTP-PE) into freeze-dried liposomes and have demonstrated their usefulness in the treatment of viral diseases. Thus, in the future, liposomes may become a more widely used vehicle for delivery of drugs in antiviral chemotherapy.

#### A.1.1.1 Liposome Targeting of Antivirals

One of the early applications of liposomes as a delivery vehicle for antivirals was made in the treatment of herpes keratitis with iododeoxyuridine (31). In this study, liposome encapsulated iododeoxyuridine was shown to be more effective than free drug in the treatment of both acute and chronic keratitis. In another study Kende et al.(32) showed a five-fold increase in liver concentrations of ribavirin following liposome encapsulation. In addition, encapsulated drug was more effective than free drug in the treatment of mice infected with the hepatotropic Rift Valley fever virus. More recently, we have demonstrated a five-fold increase in pulmonary concentrations of ribavirin when drug was encapsulated in liposomes and administered intravenously (20). Liposome-encapsulated ribavirin was more effective than free drug in protecting mice against lethal challenge with influenza virus. Thus, tissue targeting of antivirals with liposomes substantially increases their therapeutic index.

#### A.1.1.2 Liposome Targeting of Immunostimulants

Liposome encapsulated immunomodulators such as macrophage activating factor (MAF) and muramyl dipeptide (MDP), or its lipophilic analogue (MTP-PE), have been used to stimulate pulmonary macrophage activity and enhance host resistance to lung metastasis

(26,33,34) and herpesvirus infection (19,20). Koff et al. (19) have shown that liposome-encapsulated MTP-PE was more effective than free MTP-PE in preventing death in a murine model of HSV-2 hepatitis. Similarly, we have demonstrated that liposome encapsulated MTP-PE was more effective that free drug in a murine model of HSV-1 pneumonitis (20). Protection observed with liposome encapsulated drug appeared, in part, to be due to enhanced drug localization in the liver and lungs of infected mice. In another study, it was shown that liposome-encapsulated MTP-PE was more effective than free drug in the healing of genital lesions induced by HSV-2 infection of guinea pigs. Therefore, liposome encapsulation of immunostimulants can be used to enhanced localization of drugs in various tissues, thus providing an effective stimulus for the activation of local macrophages.

### A.1.2 Drug/Glycoprotein Conjugates

Drug conjugated glycoproteins or neoglycoproteins (proteins such as serum albumin to which sugars have been attached) are promising targeting vehicles because they are easily prepared, biodegradable, and nontoxic. Fiume et al. (35-37) have shown that drugs can be stably conjugated to asialofetuin or galactosyl-terminating serum albumin and bind to lectin receptors on hepatocytes (38,39). Selective uptake of these intravenously administered drug conjugates resulted from the binding of carrier to galactosyl binding sites on hepatocyte membranes (35). Quite remarkably, drugs conjugated to serum albumin were selectively cleared by neighboring liver sinusoidal endothelial and Kupffer cells (37). Once inside these cells, the drug-conjugates entered lysosomes where hydrolytic cleavage released the active drug into the cytoplasm. In a similar study Monsigny et al. (40) were able to demonstrate the targeting of methotrexate by conjugation to fucosylated neoglycoproteins. These conjugates resulted in a more efficient killing of tumor cells by methotrexate.

As previously indicated, before a carrier can be effective, the link between drug and carrier must remain stable in the bloodstream and withstand the action of serum hydrolases. On the other hand, unless the drug is able to act in conjugated form at the cell surface, it must be released from the carrier after interaction of the conjugate with the target cell. In addition, its mode of release must allow the drug to reach its subcellular target and interact effectively with it. Because the most general fate of molecules bound by surface receptors is to be internalized by endocytosis, and conveyed to lysosomes for digestion, an obvious way of insuring appropriate release of drug is to rely on lysosomal hydrolysis. This approach is of course limited to drugs that are not inactivated in the lysosomes and that can reach their biochemical target from the lysosomal compartment. Monsigny et al have demonstrated that the stability of daunorubicin-carrier conjugates in serum can be enhanced by incorporating peptide spacers between this drug and its carrier (41). These "drug-arm-carrier" conjugates can be specifically cleaved by lysosomal proteases leading to the release of active drug inside target cells. Moreover, these conjugates were more effective than free drug in killing of tumor cells. Similar results were obtained by Trouet et al. (16) using oligopeptide spacers varying in length from one to four amino acids. In their studies, the direct conjugate between daunorubicin and carrier was resistant to hydrolysis by lysosomal enzymes and drug was not released intracellularly in an active form. Hydrolysis by lysosomal enzymes and subsequent drug activity was, however, increased following introduction of a tetrapeptide spacer. The tetrapeptide conjugates remained stable in the presence of serum, as required for authentic lysosomotropic drug-carrier complexes, and the chemotherapeutic activity of daunorubicin increased with the carrier's sensitivity

to lysosomal hydrolysis. Similar augmentation in the therapeutic index of primaquine was observed in both extraerythrocytic and hepatic stages of murine malaria following linkage of this antimalarial agent to a hepatocyte-targeted glycoprotein via a tetrapeptide spacer (42).

#### A.1.2.1 Glycoprotein Targeting of Antivirals

Fiume et al. (43) have demonstrated that galactosylated serum albumin conjugated to adenine arabinoside (ara-a) and asialofetuin-ara-a conjugates, administered to mice with ectromelia virus-induced hepatitis, were equally effective in inhibiting virus DNA synthesis in liver without producing significant inhibition of cellular DNA synthesis in intestine and bone marrow. Similar results were observed with ara-c and trifluorothymidine (35-38,44). These findings were, in part, due to selective hepatocyte uptake which in turn reduced the whole animal dosage required for a therapeutic effect (i.e, ten-times less conjugated ara-a was required to inhibit viral DNA synthesis as compared to free ara-a). Galactosylated serum albumin has a significant advantage over asialofetuin as a hepatotropic carrier since conjugates prepared with homologous albumin are not immunogenic (43).

#### A.1.2.2 Glycoprotein Targeting of Immunostimulants

A number of synthetic immunostimulants are active in vitro, however, due to pharmacological problems, their in vivo activity is minimized. One approach to overcome this problem has been to conjugate immunostimulants to glycoproteins or neoglycoproteins. For example, Monsigny et al. (45) and Roche et al. (46) have shown that MDP-conjugated mannosylated neoglycoproteins can be targeted to alveolar macrophages. These macrophages become tumoricidal and mice and rats are protected against metastatic growth of Lewis lung carcinoma. MDP-conjugated mannosylated neoglycoprotein are several orders of magnitude more effective than free MDP in activating macrophages and protecting animals. Recently, Monsigny has conjugated MTP to polylysine partially substituted with gluconyl groups. These conjugates are even better than the neoglycoprotein conjugates in potentiating macrophage activity and are currently in phase I clinical trials in France (see Methods section below).

### A.2 Combination Antiviral Therapy

Experience with anticancer chemotherapeutic agents has clearly demonstrated that combination therapy is often more effective than the additive effects of individual agents. This is particularly apparent when the mechanisms of action of drugs are different. As the number of antiviral agents increases, it is reasonable to believe that similar combination therapy will be effective in viral diseases. Combination therapy offers a distinct advantage over single agent therapy in that the therapeutic dose can be reduced

and toxicity minimized. Moreover, the number of potential failures or recurrences due to the selection and overgrowth of drug resistant virus mutants can be limited. A number of observations on antiviral drug combinations with either additive or synergistic effects have been reported (47-55). For example, Fischer et al. have demonstrated the in vitro synergistic effects of 5'-amino-thymidine and iododeoxyuridine against herpes simplex virus (49). Ayisi et al. have shown that combinations of 5-methoxymethyl-deoxyuridine with either vidarabine or phosphonoacetic acid are synergistic against herpesviruses in cell culture (48). In addition, vidarabine in combination with phosphonoacetic acid has been found to produce a synergistic response against herpesviruses and retroviruses invitro (51) and against herpesviruses in vivo (52). Combinations of acyclovir with either vidarabine, vidarabine monophosphate or phosphonoformic acid have been reported to produce additive antiviral effects both in vitro and in vivo (53,54). Similar enhancement of activity has been achieved with combinations of antiviral agents against human influenza viruses in vitro (50). The combination of ribavirin with either amantadine or rimantadine hydrochloride resulted in a significantly enhanced antiviral effect against a several subtypes of influenza A viruses. Recently, combination therapy with azidothymidine and recombinant interferon alpha resulted in synergistic inhibition of human immunodeficiency virus replication (55).

## B. OVERALL OBJECTIVES AND APPROACH

### B.1 Phase I Studies

#### B.1.1 Broad Spectrum Activity of Liposome-Encapsulated Drugs

Our previous studies have demonstrated the effectiveness of liposomes as carriers of antiviral and immunostimulating drugs in the treatment of herpes- and arenavirus infections. The general acceptance of liposomes as carriers of antiviral and immunostimulating drugs will depend, in part, on the demonstration of their effectiveness in a variety of viral diseases. Thus, we will extend our previous DOD contract studies to include additional murine models of diseases of interest to the military, i.e., the bunyavirus, Punta Toro, and the flavivirus, Banzi. We will also study murine models of retrovirus-induced immunosuppression (e.g. Rauscher leukemia and LP-BM5 virus-induced murine AIDS). These viruses were selected because of their different tissue-tropisms (i.e., Punta Toro for liver, Banzi for brain and Rauscher and MAIDS for lymphoid cells). In addition, Punta Toro and Banzi viruses result in acute infections whereas Rauscher and LP-BM5 viruses result in chronic persistent disease.

The effects of chemotherapeutic and immunotherapeutic agents will be assessed by measuring mean survival time, viremia, and/or virus replication in selected organs. Survivors will be examined for virus specific serum antibody and resistance to virus rechallenge . The Punta Toro and Banzi virus models are currently in use in our laboratory. Rauscher leukemia and LP-BM5 viruses have been used as models for screening of antivirals effective against AIDS, a disease of worldwide significance. Studies on these viruses have been conducted in collaboration with Dr. Erik De Clercq of the Rega Institute, Leuven, Belgium. One of us (JDG) spent part of his sabbatical (1987-1988) in Dr. De Clercq's laboratory during which time he gained experience with several retroviral models of immunosuppression. Both the murine AIDS and Rauscher leukemia models are now established in our laboratory.

Our initial studies have focused on ribavirin and MTP-PE since these drugs were effective in our other models. However, subsequent studies will include additional antivirals and/or immunostimulants. The selection of these antivirals and immunostimulants will be made in consultation with the contracting officer.

### B.1.2 Selective Drug Targeting by Neoglycoproteins

We have demonstrated in studies supported by previous DOD contracts that liposomes are useful vehicles for the delivery of antivirals and immunostimulants. However, these vehicles have some limitations as targeting agents in that they are compartmentalized primarily by the reticuloendothelial system. Therefore, a second targeting approach has employed conjugation of drugs to neoglycoproteins. This approach has allowed us to deliver drugs to more specific sites of viral infection. Initially, our studies have focused on ribavirin and MTP-PE since these have proven effective following liposome encapsulation. Subsequent studies will employ additional antivirals and immunostimulants which will be selected in consultation with the contracting officer.

One of our goals for the first year of this contract was to determine the effectiveness of conjugated drug delivery in the treatment of virus infections which occur at different tissue sites. Future studies will compare the relative effectiveness of different targeting approaches using both murine and guinea pig models of viral diseases described in our contract proposal. Successful therapeutic approaches will then be extended to the nonhuman primate models of influenza and Punta Toro previously described.

Homologous serum albumin with added terminal mannose or galactose residues will be conjugated with drugs to target them to macrophages and hepatocytes, respectively. Drug conjugates will be chemically characterized to determine the amount of drug per

carrier molecule and biologically characterized to determine functional activity. Conjugation of antivirals with galactosylated albumin should provide a more efficient delivery to infected hepatocytes while conjugation of immunostimulants to mannosylated albumin should enhance the uptake by macrophages and monocytes. Dr. Monsigny from the Universite' d'Orleans, Centre de Biophysique Moleculaire du Centre National de la Recherche Scientifique, Orleans, France, will be collaborating with us in the preparation and characterization of drug-conjugated proteins. Dr. Monsigny has had extensive experience in conjugating drugs such as muramyl dipeptide and tripeptide to neoglycoproteins. In addition, he has recently developed a delivery system consisting of poly-lysine partially substituted with gluconyl groups which is more a more effective carrier than serum albumin. This carrier has recently received approval by the French medical authorities for use in man.

### B.1.3 Combination Antiviral/Immunostimulant Therapy

A number of newly developed drugs show promising antiviral activity in vitro. However, in vivo, many of them cause adverse side effects including damage to components of the immune system. Thus, while antivirals may reduce viral burdens in target organs, a host with a compromised immune system may not effectively remove residual virus. Therefore, any damage to immune components should be compensated for during antiviral chemotherapy. Our approach to this problem will be to attempt to balance the immunosuppressive effects of antivirals with administration of immunostimulants. This strategy has the added advantage that immunostimulants may also act synergistically in those situations where antivirals have minimum or no immunosuppressive effects. Our preliminary data (Contract DAMD 17-84-C-4144 and J. Cell Biology, Supplement 12B, 1988, Abstract W-102 page 255) clearly indicates that combination antiviral/ immunostimulant therapy is indeed effective in the treatment of herpesvirus and arenavirus infections.

Most immunostimulants possess a unique set of immunomodulating features and provide varying degrees of benefit to the infected host depending on the tissue site and degree of virus infection. For example, some immunostimulants activate macrophages and induce high levels of interferon, whereas others activate macrophages but are poor inducers of interferon. Thus, the judicious selection of an immunostimulant to be used in combination therapy with an antiviral requires 1) an understanding of the biological effects of the immunostimulant on the components of the immune system, 2) a knowledge of the mode of action of the antiviral agent, and 3) an understanding of the immune response to the virus infection.

Initially our attention will focus on combination therapy using ribavirin and MTP-PE in murine, guinea pig and primate models of disease since the effects of these drugs have already been characterized in our earlier studies. As other promising

antivirals and immunostimulants are developed and characterized under DOD contracts, we will attempt to enhance their efficacy either alone or in combination by targeting using liposomes, or neoglycoproteins. In these studies we will select immunostimulants which have a broad spectrum effect on both specific and nonspecific components of the immune system. Murine, guinea pig and primate models will be used to evaluate therapeutic potential of selected drugs.

## B.2 Phase II Studies

### B.2.1 Preclinical Evaluation of Liposome-Encapsulated Ribavirin and MTP-PE

In our previous studies we have shown that encapsulation of ribavirin and/or MTP-PE into liposomes resulted in improved targeting and therapeutic effectiveness when examined in both murine and guinea pig models of viral disease. While these results are encouraging, they do not necessarily ensure the usefulness of liposome-encapsulated drugs in viral diseases of man. A more reliable predictor of clinical usefulness should come from studies performed in nonhuman primates. Thus, in this renewal we propose to study whether targeting of antivirals and/or immunostimulants will enhance the therapeutic efficacy of these drugs in viral diseases of animal species more closely related to man (squirrel and African green monkeys). Since the liver and lung appear to be important target organs in the pathogenesis of several virus infections, we will focus our attention on infections occurring in these organs.

One of the viruses to be examined in these animals will be influenza. In this model animals will be challenged intranasally with virus and examined for fever and virus shedding. Antivirals and immunostimulants will be administered individually or in combination as well as in free or liposome-encapsulated form. Since free ribavirin has been shown to be effective in the therapy of influenza in squirrel monkeys, our studies with this drug will be designed to improve the therapeutic index of this drug via targeted delivery and/or combination with an immunostimulant.

The hepatotropic Punta Toro virus will serve as a second model of viral disease. In this model, African Green monkeys will be challenged with virus and treated with free or liposome-encapsulated antivirals and/or immunostimulants. Animals will be examined for viremia and hematological and liver enzyme changes.

### B.2.2 Timing of Phase II Studies

Phase I studies will allow us to compare the efficacy of liposomes and neoglycoproteins as targeting vehicles for antivirals and/or

immunostimulants in various viral disease models. These models provide us with an opportunity to examine the effectiveness of targeted drug delivery in both acute and chronic virus infections in which the primary sites of replication or dissemination vary. Phase II studies will allow us to test targeting approaches, proven to be effective in Phase I studies, in nonhuman primate models of viral disease. These studies should enable us to more accurately predict their clinical potential in man.

Our initial studies using liposomes as a targeting vehicle for ribavirin and/or MTP-PE have already proven successful in several of the murine and guinea pig models of disease. Thus, we will begin Phase II studies using liposome-encapsulated ribavirin and/or MTP-PE immediately. When other targeting vehicles and/or drugs prove successful in Phase I studies they will then be tested in Phase II studies. In this way Phase I and Phase II studies will be conducted concurrently throughout the contract period.

## II. RESULTS FROM YEAR ONE OF CONTRACT

The first year of this contract has been devoted to the establishment of new animal models for drug evaluation and to the production of sufficient amounts of conjugated antivirals and immunomodulators which can be tested in *in vivo* models of viral disease. More specifically, the initial year of this contract has focused on the following:

1). Development of the LP-BM5 (Murine AIDS) virus model.

2). Development of an *in vitro* viral cytopathic assay for evaluation of antivirals and immunomodulators in human macrophages.

3). Establishment of a dengue virus squirrel monkey model for the evaluation of antiviral agents.

4). Use of squirrel monkeys as a model animal for the evaluation of immunomodulating agents.

5). Antiviral profile of a new recombinant human interferon hybrid (rhuIFN-alpha B/D), from CIBA-GEIGY.

6). Conjugation of MDP, ribavirin and phosphonylmethoxyethyl adenine (PMEA) to mannosylated bovine serum albumin or polylysine carriers.

7). Evaluation of the therapeutic value of MDP-conjugates in murine models of viral pneumonitis and hepatitis.

8). Immunostimulating properties of MDP-BSA-Mannose and MDP-Polylysine-Mannose: Effects on RES function, macrophage phagocytosis and cytotoxicity.

9). Evaluation of the antiviral potential of ribavirin and PMEA polylysine carriers in virally infected human macrophages.

### 1- Murine AIDS Model

Together with CIBA-GEIGY scientists, we have developed a method to quantatively assess lymphadenopathy and splenomegaly using nuclear magnetic resonance (NMR) imaging. The advantage of this technique is that mice do not have to be sacrificed and can be monitored over the entire course of the disease. An example of the NMR images produced by this method is shown in Figure 1. Note the clarity of parathymic, axial and inguinal lymph nodes and spleen. Automated analysis of these structures provides the means with which node and

spleen size of individual animals can be objectively determined over a prolonged period of therapy.

In addition to NMR analysis of internal organs, we have also developed an in vivo virus challenge model in which immunosuppressed mice become susceptible to lethal infection with HSV-1. This has been a very valuable adjunct measurement of the degree of suppression and has allowed us to gain further insight to the value of antiviral agents to slow or prevent immunosuppression from occuring. Table 1 illustrates the effects of HSV-1 superinfection on LP-BM5 immunosuppressed mice. This enhanced susceptibility provides an opportunity to study therapeutic intervention in a murine model of retroviral-induced immunosuppression as well as on HSV-1 superinfection. Another feature of this model which has important implications is our discovery that adherent peritoneal cells obtained from LP-BM5 virus infected mice are able to transmit infection. This indicates that macrophages are infected at some time during the course of this disease (Table 2).

The effectiveness of experimental anti-herpes and retroviral agents administered to immunosuppressed mice prior to and during HSV-1 superinfection has been examined. This data is presented in the manuscript entitled "9-(2-Phosphonylmethoxyethyl) Adenine (PMEA) in the Treatment of Murine Acquired Immunodeficiency Disease (MAIDS) and Opportunistic Herpes Simplex Virus Infections" which has been included in Appendix I. It should be noted that some of the MAIDS developmental studies were performed while one of us (JDG) was working in Basel, Switzerland during the first year agreement of our contract.

## 2- Human Monocyte Antiviral Assay

Development of an in vitro assay for the evaluation of antivirals and immunomodulators which have been chemically modified (ie. conjugated to glycoproteins or encapsulated into liposomes) is needed to ensure that the biological activity of these drugs has not been reduced or destroyed. Moreover, it is important that cells used in the in vitro assay system are relevant to the targeting approach which we are using. A second manuscript, "Use of Human Monocytes for the Evaluation of Antiviral Drugs: Quantitation of HSV-1 Cytopathic Effects" describes this procedure and is included in Appendix II of this report. This manuscript provides a through description of the methodology used to assay antivirals and immunomodulators in cultured human macrophages. Several drugs, including poly I:C-LC, rhuIFN-alpha B/D, acyclovir and ribavirin have been evaluated in this assay (See Appendix II). Poly I:C-LC, rhuIFN-alpha B/D and acyclovir were highly effective in protecting macrophages from the cytopatheic effects of HSV-1 infection. In contrast, ribavirin had little or no effect. We are

currently examining the ability of other viruses such as Pichinde (AN 3739), Punta Torc, Sandfly Fever and Influenza (Aichi) viruses to cause a cytopathic effect in these macrophages. If successful, these will be incorporated into our antiviral assays.

3- Development of a dengue virus squirrel monkey model

A primate adapted dengue type 1 virus (West Pac 74) was obtained from Dr. Ken Eckles (WRAIR Wash. DC) and used to infect squirrel monkeys, *Saimiri sciureus*. This virus was supplied in lyophilized form and was reconstituted in pyrogen free water prior to intramuscular inoculation (0.5 ml, approximately 5 X $10^4$PFU) into the upper forearm. Dengue virus was inoculated into male *S. sciureus* monkeys which were screened for the presence of neutralizing anti-dengue antibodies by Dr. Ken Eckles. Animals were examined daily for viremia, fever, liver enzyme and differential white blood cell counts. Preliminary data are now being evaluated and we hope to have this dengue primate model established by the end of this summer.

4- Use of squirrel monkeys as a model for evaluation of immunomodulating agents

Squirrel monkeys were held in our animal quarters for several days prior to being bled to determine baseline serum interferon levels. These monkeys were gavaged with 25 mg/kg of CL 246,738 and their serum collected each day for three days. Gavage (25 mg/kg) was repeated on day four and serum samples collected on days five and seven. All serum samples were analyzed for interferon levels in primary human foreskin cells and VSV as the challenge virus. Cell destruction was evaluated using the neutral red dye uptake and the colorimetric assay described in the attached manuscript (See Appendix II) . Briefly, serial (three-fold) dilutions of serum were added to foreskin monolayers in microtiter plates and the plates were incubated for 18 hours. Treated cells were challenged with VSV and incubated for another 48 hours prior to the addition of neutral red. Note that in Table 3, the dilution of the NIH international human interferon standard (10,000 units by plaque reduction) was 4706 by this assay.

Three monkeys were examined for serum interferon levels. All had maximal responses at day two post inoculation; monkey three had the highest interferon response (260 U/ml = 520 IU/ml) and monkey two the lowest (78 U/ml). Unexpectedly, none of the three responded to a second gavage (CL 246,738, 25 mg/kg) administered four days after the first.

5- Antiviral Profile of rhuIFN-alpha B/D

RhuIFN-alpha B/D is a human hybrid interferon which is cross-species active and can be utilized in a variety of animals. Figure 2 illustrates the potent antiviral activity of rhuIFN-alpha B/D compared to alpha A (Roferon) or gamma interferons in human monocytes infected with HSV-1. Note the lack of gamma activity and potent rhuIFN-alpha B/D response.

The in vivo antiviral activity of rhuIFN-alpha B/D in murine models of HSV-1 pneumonitis (VR3) and encephalitis (MacIntyre) are presented in the manuscript entitled "Antiviral activity of a human recombinant interferon alpha B/D hybrid" (Appendix III). The results from these studies are quite impressive considering that treatment was initiated several hours after infection. Likewise, similar efficacy was demonstrated in the Friend murine leukemia and LP-BM5 models. Note that rhuIFN-alpha B/D was effective in slowing the onset of virus-induced immunosuppression (as measured by mitogen response), splenomegaly and lymphadenopathy when administered subcutaneously every second day (50 million units/kg) for 14 days following infection. Moreover, when administered together with suboptimal doses of AZT, rhuIFN-alpha B/D was able to augment the effects of AZT. Similar augmentation was observed when other nucleoside analogues were used together with rhuIFN-alpha B/D (See Appendix I).

## 6- MDP and Ribavirin Conjugates

The procedures used to conjugate antivirals and immunopotentiators to BSA or poly-L-lysine are as follows. In the first step, mannosylated BSA was prepared by allowing a-D-mannosyl-pyranosyl-phenyl-isothiocyanate (MPPI) to react with BSA according to a method derived from McBroom, Samanen and Goldstein (Meth. Enzymol., 28:212, 1972). Briefly, 301.5 mg (1 mmol) of p-nitrophenyl-a-D-manno- pyranoside (Sigma) was dissolved in 20 ml of 4:1 methanol:water mixture. To this solution was added 30 mg of 10% Palladium on charcoal (Merk). The suspension was stirred under hydrogen (1 atm) at room temperature for two hours and then filtered. The filtrate was evaporated under reduced pressure at 40 C. The p-nitrophenyl- a-D-manno-pyranoside thus obtained was dissolved in 30 ml of 0.1 M sodium carbonate, pH 8.6. To this solution was added 30 ml of chloroform containing 2 mmoles of thiophosgene. This mixture was shaken for 30 minutes and the a-D-manno-pyrannosyl-phenyl- isothiocyanate thus formed was crystallized. The crystals were washed in chloroform and cold distilled water. 31 mg of recrystallized MPPI was added to 25 ml of a solution containing 268 mg BSA in 0.1 M sodium carbonate (pH 9.5). After 20 hour stirring , the resulting manno-pyrannosyl-thiocarbamyl serum albumin (Man-BSA) was purified by gel filtration on an Ultrogel-202 column in distilled water. The mannose content of Man-BSA was determined by the resorcinol-sulfuric acid method.

MDP serum albumin conjugate was prepared by adding MDP hydroxysuccinimide ester to a solution of BSA or Man-BSA in 1 M sodium bicarbonate buffer, pH 8.5. MDP-hydroxysuccinimide ester was prepared by dissolving 100 mg (0.2 mmole) MDP, 46 mg (0.24 mmole) dicyclohexyl- carbodiimide and 26.6 mg (0.24 mmole) N-hydroxysuccinimide in 2 ml of freshly distilled dimethyl formamide. After 24 hours at 25$^{o}$ C, MDP was quantitatively converted into MDP-hydroxysuccinimide ester. The solution of MDP-hydroxysuccinimide ester in dimethylformamide was added to 17 ml solution of 170 mg of Man-BSA or BSA in 1 M sodium bicarbonate buffer at pH 8.5. The reaction mixture was stirred for 24 hours at room temperature. The conjugate was purified by gel filtration on Ultrogel 202 column in phosphate buffered saline, pH 7.4. The MDP content of the conjugate was determined by the method of Levy and McAllan (Biochem. J., 73:127, 1959). Similar procedures were used in the conjugation of ribavirin to poly-L-lysine and BSA.

Table 4 lists the MDP and Ribavirin BSA and Poly-L-Lysine conjugates prepared by Dr. Michel Monsigny in Orleans, France and currently under study in our laboratory. Four of these conjugates (ie. MMB 350, MMB 351, PLL Mannose, and MDP-PLL-Mannose) have been studied in detail (see below). A structural drawing of the MDP-Poly-L-Lysine- Mannose (MDP-PLL-Mannose) and Ribavirin-PLL-Mannose conjugates is presented in Figure 3. Note that free amino groups are blocked with carbohydrate groups to prevent nonspecific cellular attachment and rapid clearance from the circulation. The MDP linkage illustrated in Figure 3 is highly stable and has been successfully employed by Dr. Monsigny for a number of years. Several variations in the chemical linkage of Ribavirin to poly-L-lysine (eg. the addition of glycyl-glycine spacers) are under investigation in Dr. Monsigny's lab. These studies will enable us to select the optimal drug linkage for stability and biological activity.

## 7- MDP Conjugates in Murine Models of HSV-1 Induced Pneumonitis and Hepatitis

Figure 4A illustrates the therapeutic activity of MDP-BSA conjugates in a murine model of herpesvirus (HSV-1) hepatitis. In this infection model, virus is administered intravenously and within 2-3 days results in viremia and fulminant liver infection. Note that when drug was administered intravenously (10 ug/mouse, 2 days prior to infection, on the day of and two days following infection), MDP-BSA-Man-6-$PO_4$ (MMB 351) was highly effective in preventing death and in enhancing mean survival times. A similar effect was observed with MDP-BSA (MMB 350) (data not shown). MDP-BSA-Man-6-$PO_4$ (administered i.v., 10 ug/mouse as above) was more effective than free MDP in prolonging the mean survival time of mice infected intranasally with a strain of HSV-1 causing pneumonitis (Figure 5A).

Figure 4B illustrates the therapeutic activity of MDP-Poly-L-lysine-Mannose in the murine model of HSV-1 hepatitis. Note that intravenous administration of drug (10 ug/mouse) 2 days prior to infection, on the day of infection, and 2 days following infection resulted in 40% survival and prolonged the mean survival time of mice. A significant prolongation of mean survival time was also observed in mice receiving intravenous MDP-Polylysine-Mannose (10 ug/mouse) and infected intranasally with HSV-1 (Figure 5B).

## 8- Immunostimulating properties of MDP conjugates

### A. Stimulation of RES Functions

Mice treated intravenously with 10 ug of MDP-poly L lysine two days prior to the intravenous inoculation of chromium-labeled sheep red blood cells were able to clear SRBC from their circulation at a much greater rate than mice receiving carrier alone or free MDP (Figure 6).

### B. Effects on Macrophage Phagocytosis and Cytotoxicity

No significant differences in peritoneal macrophage phagocytosis was observed 2 days following a single intraperitoneal inoculation of MDP-BSA or MDP-BSA-Man-6-$PO_4$ (10 ug/mouse) (Table 5). This negative response may be due to drug dosage and/or time of administration; therefore, future experiments will examine these parameters.

Little change in macrophage cytotoxicity was observed 2 days following a single inoculation with either the BSA or Polylysine MDP conjugates (10 ug/mouse) (Tables 6 & 7). Nonetheless, there was a suggestion that cytotoxicity levels were elevated (see 20 and 10:1 effector to target cell ratios in Table 6) following administration of either MMB 350 (MDP-BSA) or MMB 351 (MDP-BSA-Man-6-$PO_4$). Future experiments will examine macrophage cytotoxicity levels at later times (ie. 3 or 4 days post inoculation) following either single or multiple drug dosages.

## 9- Evaluation of the antiviral activity of nucleoside analogue poly-lysine conjugates in human macrophages

We now have several nucleoside analogues (eg. PMEA, ribavirin and AZT) conjugated to poly-lysine. Preliminary data indicate that PMEA conjugated to poly-lysine was as good or better than free PMEA in preventing the lysis of human macrophages infected with HSV-1 (Figure 7). Free or conjugated ribavirin were not effective in preventing the cytopahtic effects of HSV-1 in human macrophages. Future studies will employ models of RNA virus infections

(Influenza, Pichinde, and Punta Toro) which may be more susceptible to the antiviral activity of ribavirin.

## III. Conclusions and Future Plans

1. The *in vivo* virus challenge studies suggest that MDP-BSA and Polyl-lysine conjugates are more effective than free MDP in enhancing resistance to HSV-1 hepatitis and pneumonitis. Moreover conjugated MDP was more effective in enhancing RES functions than was free drug.

2. While the *in vitro* macrophage function studies have not demonstrated significant changes in phagocytosis or cytotoxicity when administered two days prior to assay, additional studies employing either single or multiple drug dosages over a longer period may prove to be more effective.

3. Squirrel monkeys have been shown to be usefull in the evaluation of immunomodulating agents which induce interferon and should be a convenient model to establish the preclinical efficacy of promising new drugs.

4. The human macrophage antiviral assay is a valuable tool for *in vitro* evaluation of targeted antivirals and immunostimulants, since this is the cell to which the drug should be targeted *in vivo*. Moreover, this cell lends itself to infection by a variety of RNA and DNA viruses and allows us to easily examine the biological activity of free and conjugated drugs.

5. Conjugation of the nucleoside analogue, PMEA, to poly-lysine was accomplished without any loss of biological activity as determined by the *in vitro* HSV-1/macrophage assay. Future studies will determine the *in vivo* activity of conjugated PMEA against both RNA and DNA viral infections.

6. We expect that the dengue/primate model may be ready for the evaluation of both liposome-encapsulated or conjugated antivirals and immunostimulants by early fall of 1989.

7. Future studies will continue to address the *in vivo* efficacy of conjugated antivirals and immunostimulants; however, we will also devote more time to liposome delivery. Thus, poly I:C will be encapsulated in liposomes either alone or together with antivirals, such as ribavirin, and evaluated in our animal models.

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TABLE 1

## Susceptibility of LP-BM5 Immunosuppressed Mice to Infection With HSV-1

| Immune Status [a] | Mortality Following HSV-1 [b] Infection (dead/total) |
|---|---|
| No LP-BM5 Infection Not Immunosuppressed | 0/10 |
| LP-BM5 Infected Immunosuppressed | 12/12 |

a. As determined by Con-A mitogen response; IgM in serum; splenomegaly and lymphadenopathy 60 days post LP-BM5 infection.

b. HSV-1 (approximately $5 \times 10^5$ pfu) was administered intranasally in 70 ul and the mice observed daily for 21 days.

TABLE 2

**MACROPHAGE INVOLVEMENT IN MURINE AIDS INFECTION**

| Inoculum[a] Source | Disease Manifestations | | | |
|---|---|---|---|---|
| | Spleen Weight[b] (mg ± SD) | Con A Response[b] (SI) | Lymphadenopathy[c] | Susceptibility[d] to HSV-1 Infection (% mortality) |
| Peritoneal MØ from uninfected mice | 74 ± 13 | 6 | - | 0 |
| Peritoneal MØ from LP-BM5 infected mice | 2039 ± 760 | 0 | ++++ | 100 |
| Splenic MØ from uninfected mice | 78 ± 12 | 4 | - | 0 |
| Splenic MØ from from LP-BM5 infected mice | 1998 ± 300 | 0 | ++++ | 100 |

a. Obtained from mock or LP-BM5 infected mice 60 days post-inoculation.
b. Assays performed 60 days after LP-BM5 inoculation.
c. Mice exhibiting enlarged axial and inguinal lymph nodes. Lymphadenopathy correlated with a loss in splenic Con A response.
d. Mice were infected with HSV-1 60 days after LP-BM5 inoculation.

Table 3. Serum interferon levles in squirrel monkeys after oral administration of CL 246738 on day 0 and day 4.

| Treatment | IFN Titer on Day | | | | | |
|---|---|---|---|---|---|---|
| | Pre-Bleed | 1 | 2 | 3 | 5 | 7 |
| Monkey #1 CL 246738 | 0 | 31 | 140 | 2 | 3 | 0 |
| Monkey#2 CL 246738 | 0 | 0 | 78 | 1 | 5 | 0 |
| Monkey#3 CL 246738 | 0 | 67 | 260 | 1 | 6 | 3 |

Monkeys were gavaged with CL 246738 (25mg/kg) on day 0 and on day 4 and serum interferon levels were determined using VSV as the challenge virus in primary human foreskin cells. Cell destruction was evaluated using a neutral red dye uptake assay. In this assay, a NIH international standard (10,000 units by plaque reduction) gave a titer of 4706.

TABLE 4. CONJUGATED COMPOUNDS SUPPLIED BY DR. MICHEL MONSIGNY, ORLEANS, FRANCE

MDP-BSA CONJUGATES:

1. (MDP)11 - (BSA) (MMB 350)
2. (MDP)11 - (BSA) - (Mannose - 6 Phosphate)33 (MMB 351)
3. Ribavirin - BSA
4. Ribavirin - (Mannose - 6 Phosphate)

POLYL-L-LYSINE CONJUGATES:

1. (Mannose)35 - Poly-L-Lysine (PLL - Mannose)
2. (MDP)10 - (Mannose)40 - GG-PL
3. (MDP)47 - (Mannose)25 - GG-PL (MDP - PLL - Mannose)
4. (Ribavirin)20 - Poly-L-Lysine
5. (Ribavirin)20 - (Mannose)15 - PLL
6. (Ribavirin)20 - (Mannose)32 - PLL
7. PL - (Ribavirin)21 - (GLcA)133
8. PL - (Ribavirin)21 - (Mannose)35 - (GLcA)100
9. PL - (MDP)30 - (GLcA)136
10. PL - (Mannose)39 - (GLcA)122
11. PL - (MDP)30 - (Mannose)42 - (GLc)83
12. 5' - Succinyl - Ribavirin
13. (GLcA)110 - (PMEAGG)20 - Man 6PGG24 - (ACGG)36 - PLK
14. (GLcA)110 - (PMEAGG)20 - (ManGG)55 - (ACGG)5 - PLK
15. PLLys (GLcA)110 - (GGMan)60 - (GG Suc Rib)20
16. PLLys (GLcA)110 - (GGMan 6P)60 - (GG Suc AZT)20
17. PLLys (GLcA)110 - (GGMan 6P)60 - (GG RibTC)20
18. PLLys (GLcA)110 - (GGGMan 6P)15 - (GGG PAZTC)20

PLL = Poly-L-Lysine

GLcA = Gluconyl

Table 5. Phagocytosis by peritoneal exudate cells following intraperitoneal administration of MDP or MDP-BSA conjugates on day -2.

| Treatment | % Phagocytic Cells | | | |
|---|---|---|---|---|
| | Total | | | |
| Channel No. | 61-255 | 61-85 | 86-171 | 172-255 |
| Saline Control | 43 | 9 | 27 | 6 |
| BSA Control | 48 | 9 | 26 | 14 |
| Free MDP | 42 | 7 | 25 | 10 |
| MMB 350 MDP-BSA | 44 | 8 | 28 | 8 |
| MMB 351 MDP-BSA-Man-6-$PO_4$ | 44 | 7 | 27 | 9 |

Mice were given BSA (6.5mg/kg), free MDP (0.5mg/kg), MMB 350 (6.5mg/kg), or MMB 351 (7.3mg/kg), intraperitoneally, two days prior to assaying for phagocytosis of fluoresceinated S. aureus by flow cytometery. The total number of phagocytic cells and the percentages in the indicated channels were determined by integration. The fluorescence in channels 1-60 represents primarily autoflouorescnece while the fluorescence in channels 61-85, 86-171, and 172-255 represent dimly, intermediate and highly fluorescent cells, respectively.

The dosage of conjugate was adjusted so as to administer a equivalent amount of MDP (0.5ug/kg).

Table 6. Activation of cytotoxic macrophages by free MDP or MDP-BSA conjugates.

| Treatment | Statistics | Effector to target ratio | | |
|---|---|---|---|---|
| | | 40:1 | 20:1 | 10:1 |
| Control | CPM | 11,200 | 18,107 | 17,695 |
| MDP | CPM | 7,160 | 14,924 | 16,126 |
| | C.I. | 36 | 18 | 9 |
| | P-value | N.S. | N.S. | N.S. |
| BSA | CPM | 4,689 | 14,129 | 16,986 |
| | C.I. | 58 | 22 | 4 |
| | P-value | N.S. | N.S. | N.S. |
| MMB-350 | CPM | 4,748 | 11,999 | 14,192 |
| | C.I. | 58 | 34 | 19 |
| | P-value | N.S. | N.S. | N.S. |
| MMB-351 | CPM | 5,126 | 10,415 | 15,406 |
| | C.I. | 54 | 42 | 13 |
| | P-value | N.S. | N.S. | N.S. |
| Target cells alone: | | 5,189 | | |

Mice were injected ip with 10 ug MDP or its equivalent amount (130 ug) on a carrier (MMB-350 or MMB-351) in 0.2 ml pyrogen-free saline 2 days or 4 days before test. Control mice received the same amount of pyrogen-free saline or the carrier (BSA). Peritoneal cells were adhered to microtiter plates, nonadherent cells washed away and $4 \times 10^3$ P388 leukemia cells added to the plate. Cytotoxicity was assayed 48 hours later by pulsing the cells with $^3$H-thymidine for 16 hours. In this assay effector cells alone do not incorporate any thymidine. Cytotoxicity index (CI) was calculated by applying the formula:

$$100 \times \frac{\text{CPM in control cultures} - \text{CPM in treated cultures}}{\text{CPM in control cultures}}$$

This assay measures both cytolytic and cytostatic effector functions.

Table 7. Activation of cytotoxic macrophages by free MDP or MDP-PLL conjugates.

| Treatment | Statistics | Effector to target ratio | | |
|---|---|---|---|---|
| | | 40:1 | 20:1 | 10:1 |
| Control | CPM | 241147 | 250740 | 259691 |
| MDP | CPM | 255634 | 258579 | 229263 |
| | C.I. | -3 | 2 | 12 |
| | P-value | N.S. | N.S. | N.S. |
| PLL | CPM | 241147 | 250740 | 247038 |
| | C.I. | 2 | 5 | 5 |
| | P-value | N.S. | N.S. | N.S. |
| MDP-PLL | CPM | 268102 | 263538 | 255756 |
| | C.I. | 8 | 0.3 | 1 |
| | P-value | N.S. | N.S. | N.S. |
| Target cells alone: | | 127384 | | |

Mice were injected ip with 10 ug MDP or its equivalent amount (40 ug) on a carrier, MDP-PLL-Mannose (MDP-PLL) in 0.2 ml pyrogen-free saline 2 days or 4 days before test. Control mice received the same amount of pyrogen-free saline or the carrier (PLL). Peritoneal cells were adhered to microtiter plates, nonadherent cells washed away and $4 \times 10^3$ P388 leukemia cells added to the plate. Cytotoxicity was assayed 48 hours later by pulsing the cells with $^3$H-thymidine for 16 hours. In this assay effector cells alone do not incorporate any thymidine. Cytotoxicity index (CI) was calculated by applying the formula:

$$100 \times \frac{\text{CPM in control cultures} - \text{CPM in treated cultures}}{\text{CPM in control cultures}}$$

This assay measures both cytolytic and cytostatic effector functions.

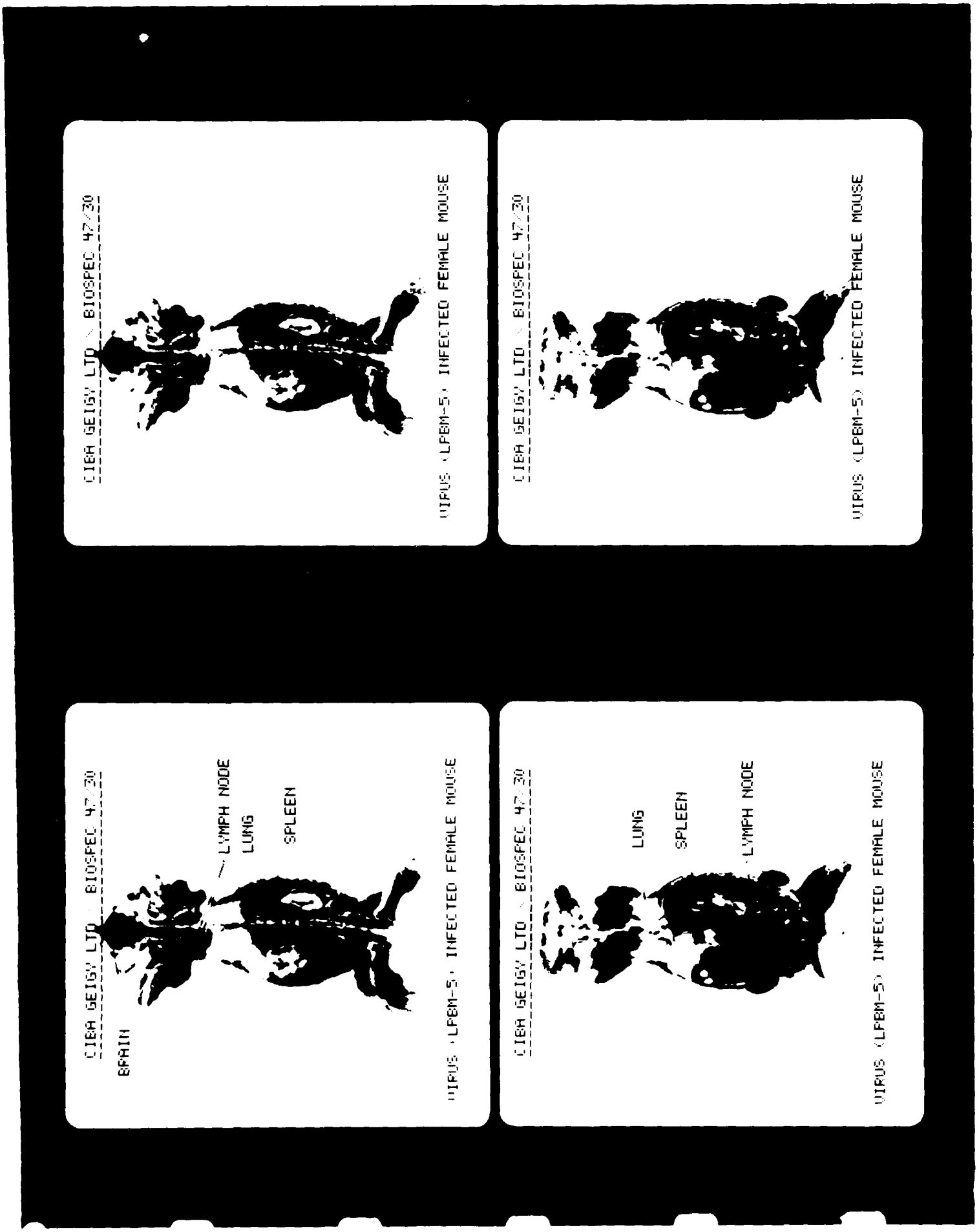
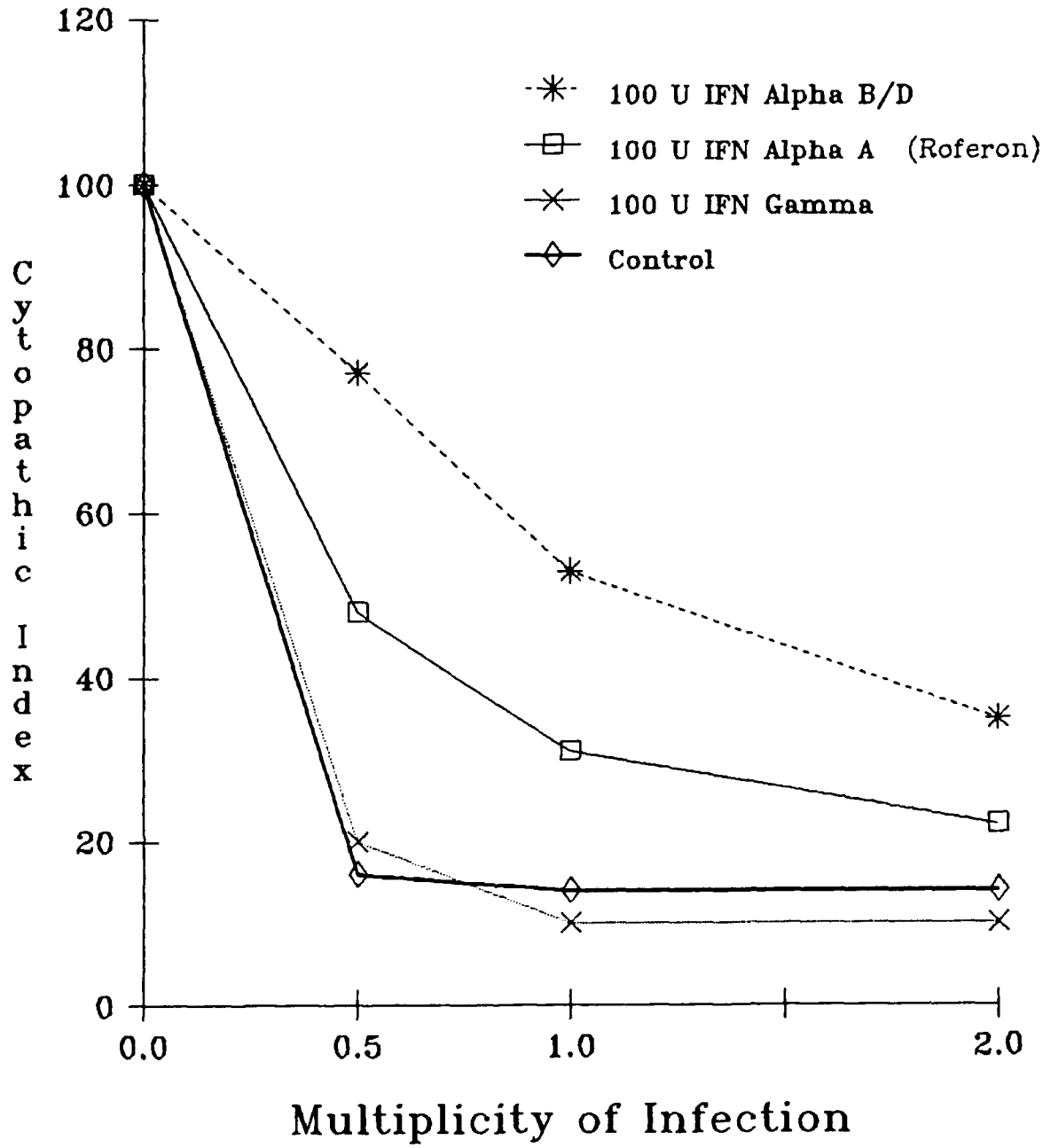

CIBA GEIGY LTD BIOSPEC 47/30
VIRUS (LPBM-5) INFECTED FEMALE MOUSE
CIBA GEIGY LTD BIOSPEC 47/30
VIRUS (LPBM-5) INFECTED FEMALE MOUSE
CIBA GEIGY LTD BIOSPEC 47/30
BRAIN
LYMPH NODE
LUNG
SPLEEN
VIRUS (LPBM-5) INFECTED FEMALE MOUSE
CIBA GEIGY LTD BIOSPEC 47/30
LUNG
SPLEEN
LYMPH NODE
VIRUS (LPBM-5) INFECTED FEMALE MOUSE

FIGURE 1

FIGURE 2

# Inhibition of HSV-1 Cytopathic Effect in Differentiating Human Monocytes

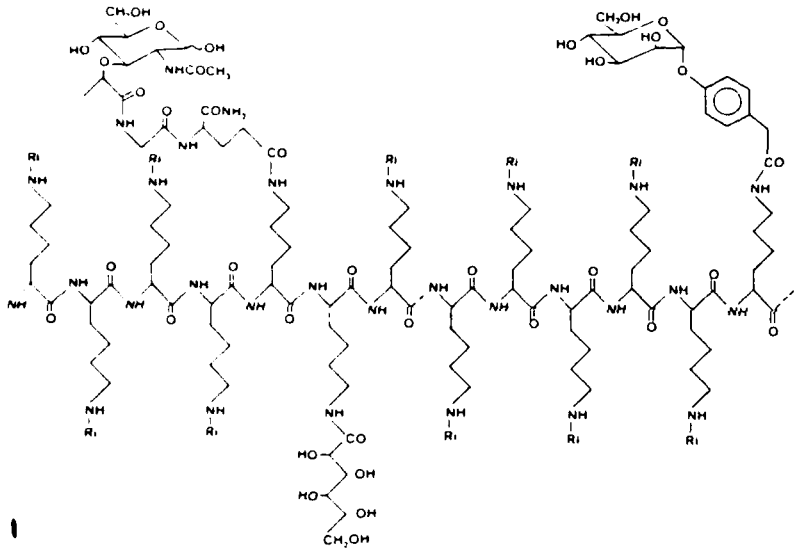



Monocytes were cultured for 10 days and then added to microtiter plates ($1 \times 10^5$ cells/well). Cells were incubated overnight with interferon or media (RPMI-1640, penn/strep, hepes, glutamine and 10% human serum) for 18 hours. Treated cells were then washed and infected with HSV-1 (VR/3). See Appendix II for complete description.

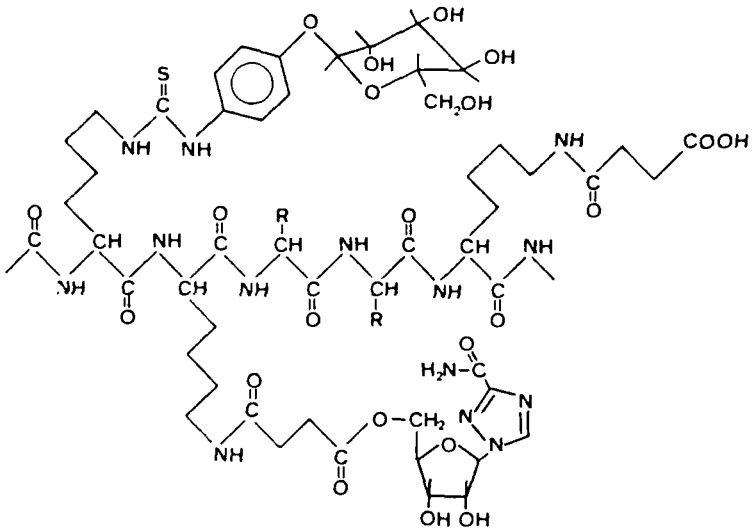
FIGURE 3

# CHEMICAL STRUCTURE OF GLUCONOYLATED AND MANNOSYLATED POLY L-LYSINE SUBSTITUTED WITH MDP OR RIBAVIRIN RESIDUES

MICHEL MONSIGNY
ORLEANS, FRANCE



(MDP)-(MANNOSE)-POLYLYSINE



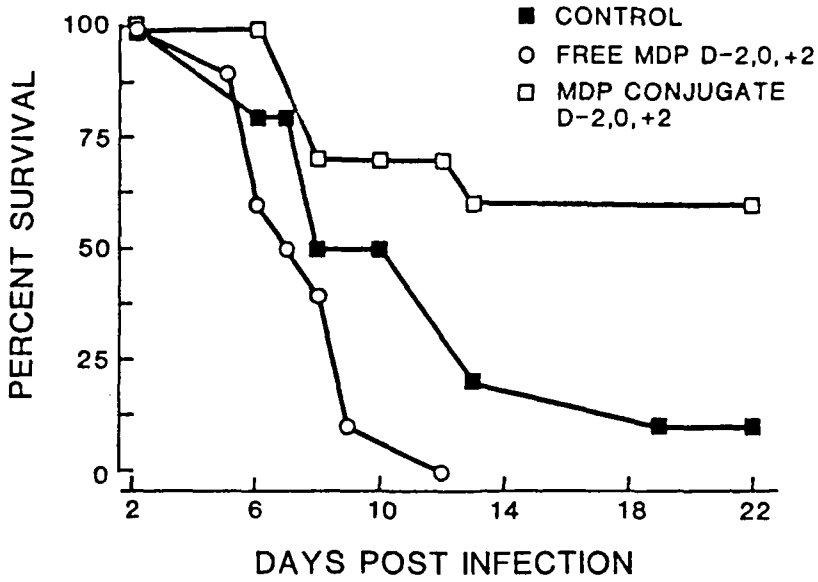
(SUCCINYL-RIBAVIRIN)-(MANNOSE)-POLYLYSINE

Ri = MDP, GlcA OR MANNOSE DERIVATIVE
R = RIBAVIRIN

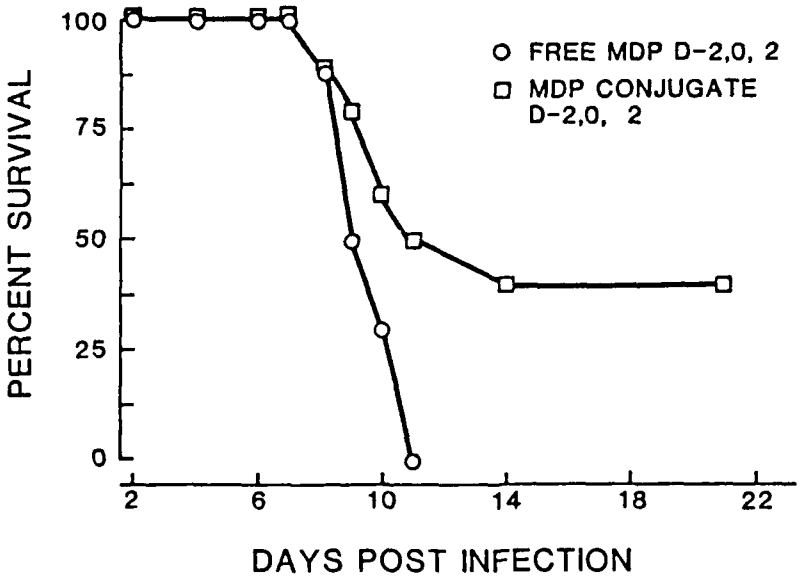
FIGURE 4

# TREATMENT OF HSV-1 INDUCED HEPATITIS WITH MDP CONJUGATES

A. MANNOSYLATED BSA-MDP (10 μg i.v.)




B. MANNOSYLATED POLY L-LYSINE-MDP (10 μg i.v.)

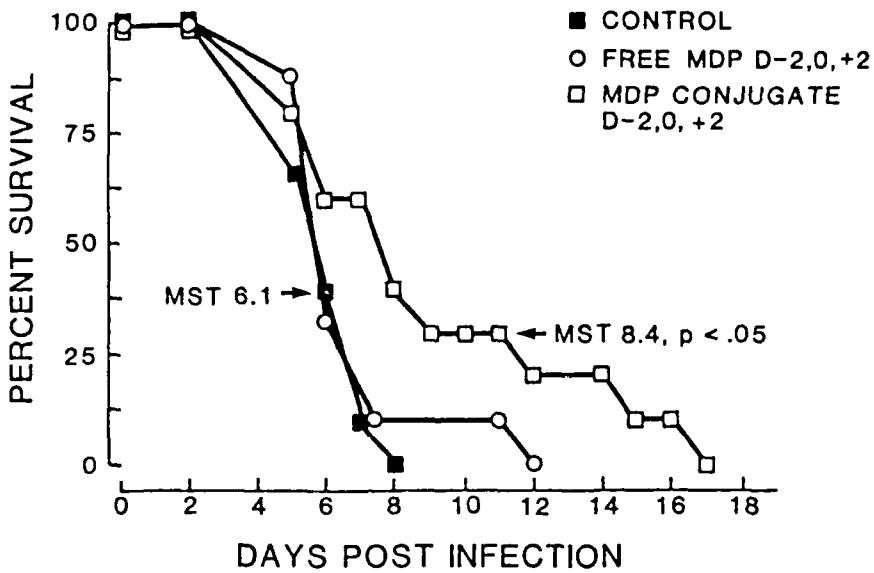



Mice received 10 ug i.v. of either free or conjugated MDP 2 days prior to, on the day of and 2 days following infection. All mice received 1 $LD_{80}$ of HSV-1 (MB strain) intravenously on day 0 and were followed for 21 days. Ten mice per group were evaluated using Wilcoxon rank analysis.

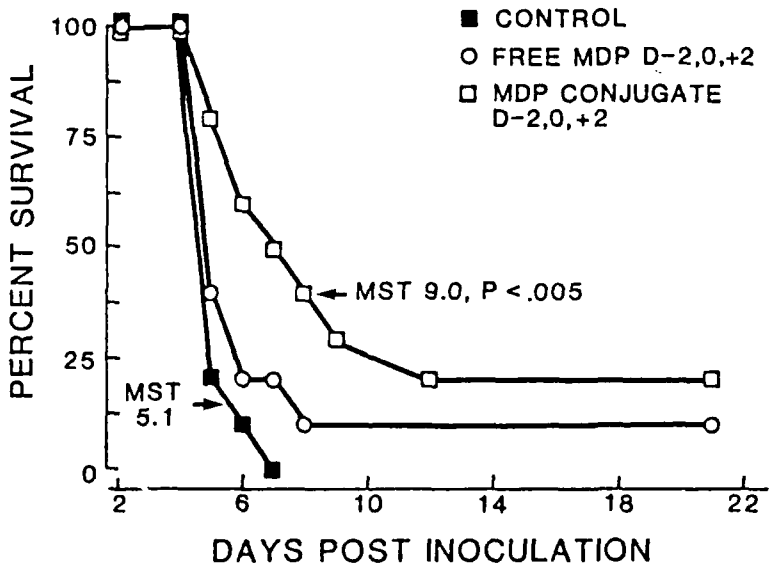
FIGURE 5

# TREATMENT OF HSV-1 INDUCED PNEUMONITIS WITH MDP CONJUGATES

A. MANNOSYLATED BSA-MDP (10 µg i.v.)




B. MANNOSYLATED POLY L-LYSINE MDP (10 µg i.v.)




Mice received 10 ug i.v. of either free or conjugated MDP 2 days prior to, on the day of and 2 days following infection. All mice received 1 $LD_{80}$ of HSV-1 (VR/3 strain) intranasally on day 0 and were followed for 21 days. Ten mice per group were evaluated using Wilcoxon rank analysis.

FIGURE 6

RES STIMULATION BY MANNOSYLATED POLY L-LYSINE MDP CONJUGATE

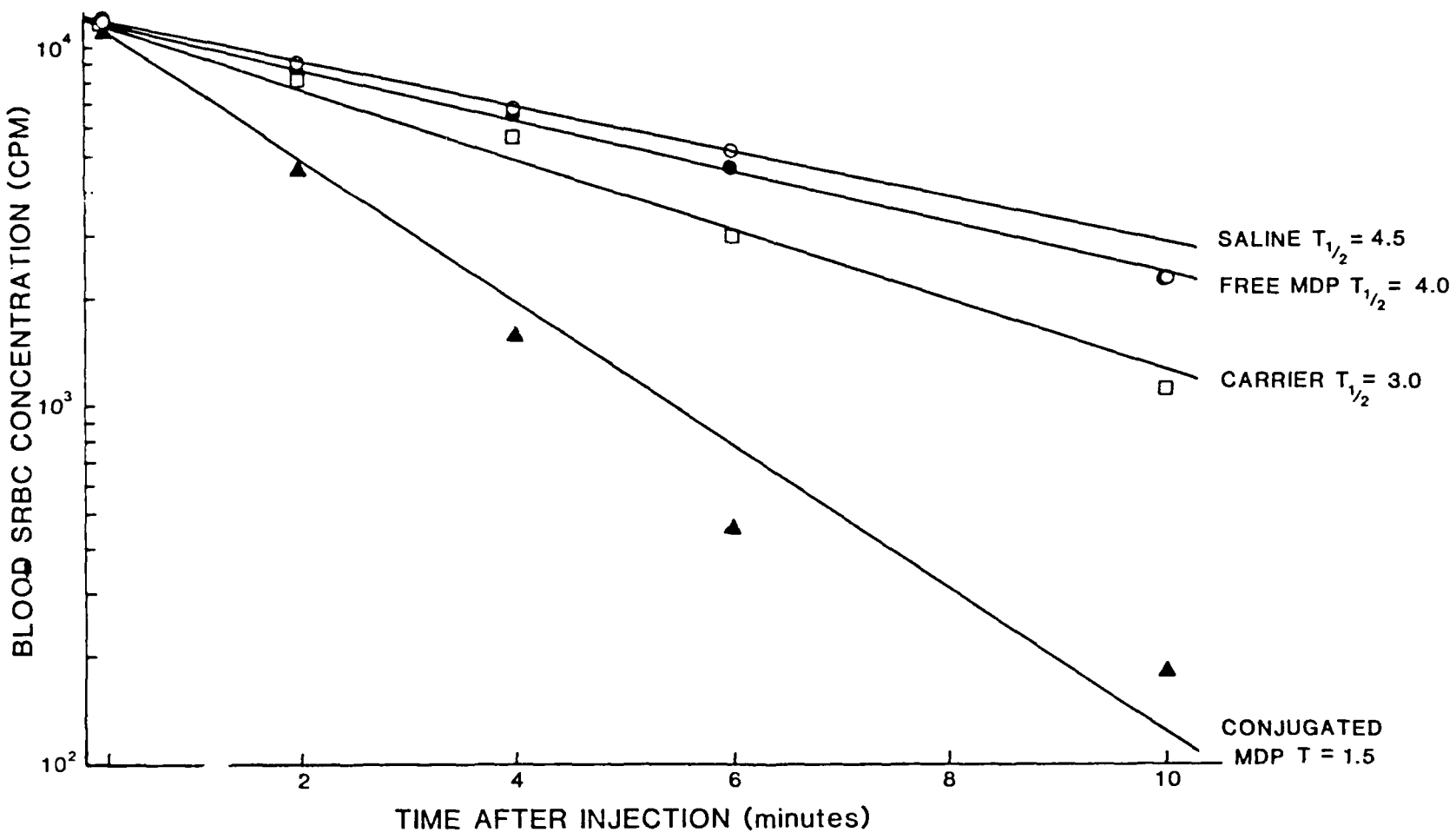
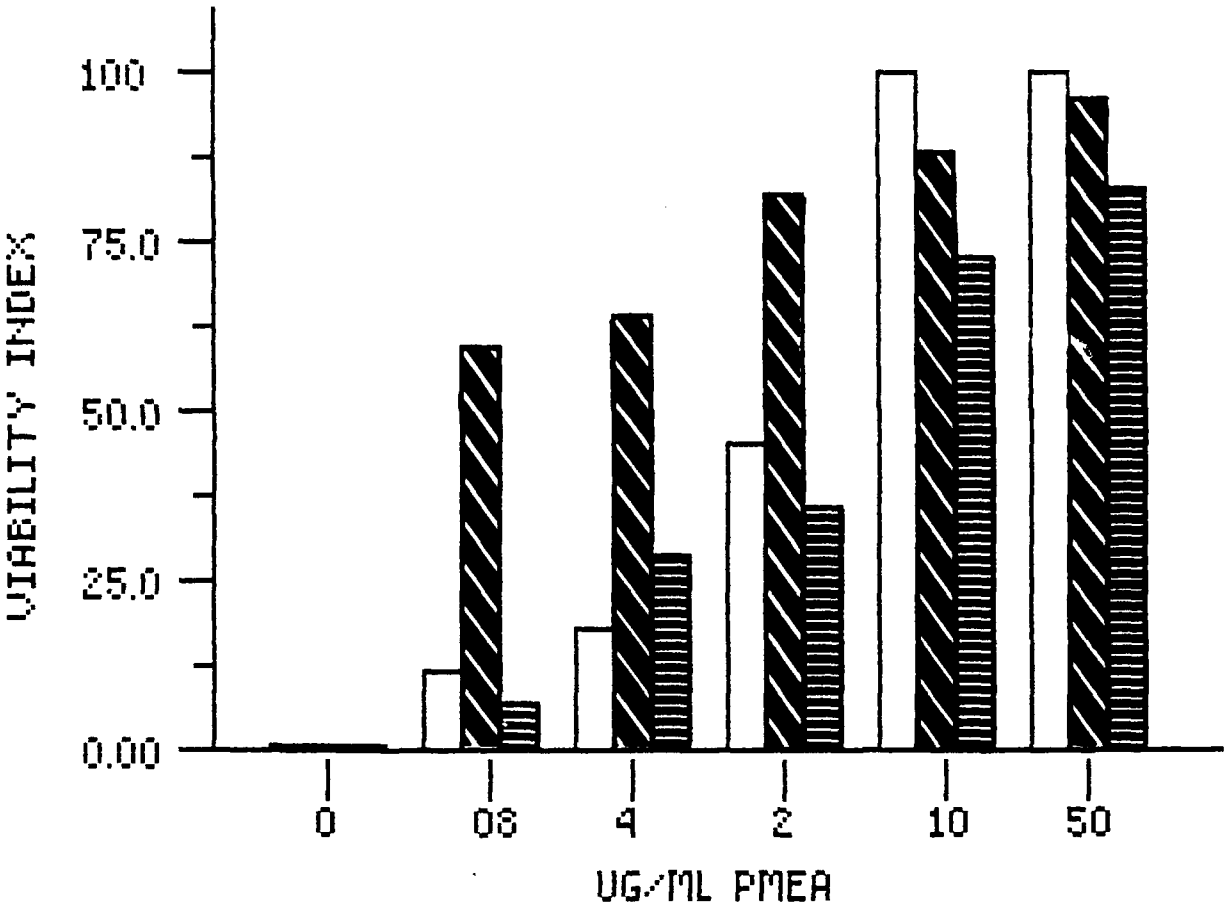


FIGURE 7

FREE VERSUS CONJUGATED PMEA IN THE
TREATMENT OF HUMAN MACROPHAGES
INFECTED WITH HSV-1




1. Free PMEA ▭.
2. Mannosylated Poly-L-Lysine PMEA ((GLcA)110 - (PMEAGG)20 - (ManGG)55 - PLK) ▧.
3. Mannose (6 PO)4 Poly-L-Lysine PMEA ((GLcA)110 - (PMEAGG)20 - (Man6PGG)24 - PLK) ▤.

Monocytes were cultured for 10 days and then added to microtiter wells ($1 \times 10^5$ cells/well). Cells were infected with HSV-1 (VR/3) at a multiplicity of infection = 1 and then incubated in the presence of drug for 24 hours. Viability was determined using neutral red dye uptake. The viability index was determined by extracting neutral red from cell monolays and then quantitating using a colorimetric procedure described in Appendix II.

APPENDIX I

APPENDIX I

# 9-(2-Phosphonylmethoxyethyl) Adenine (PMEA) in The Treatment of Murine Acquired Immunodeficiency Disease (MAIDS) and Opportunistic Herpes Simplex Virus Infections

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Running title: PMEA in the Treatment of MAIDS and Opportunistic HSV-1 Infections

## ABSTRACT

The murine model of acquired immunodeficiency disease (MAIDS) was used to evaluate both the antiretroviral and antiherpetic activity of the acyclic nucleotide analogue 9-(2-phosphonylmethoxyethyl) adenine (PMEA). The antiretroviral activity of PMEA was compared to that of azidothymidine (AZT) in mice receiving drug either immediately following infection or at late times in disease progression. Both AZT (oral, 30 mg/kg) and PMEA (parenteral, 25 and 5 mg/kg) were effective in slowing the development of disease when administered daily beginning on the day of infection. In contrast, neither drug alone was effective in modifying disease outcome when administered several weeks after viral infection. Human recombinant alpha interferon (rhuIFNalpha-B/D at $5 \times 10^7$ U/kg) was also ineffective when administered late in the course of disease. However, when administered in combination, both alpha interferon and PMEA (25 mg/kg) were able to suppress disease progression even when treatment was initiated as late as three weeks post-infection. Mice, immunocompromised due to LP-BM5 virus infection, are highly susceptible to acute (lethal) infection with HSV-1, while age-matched immunocompetent littermates are not. PMEA was as effective as acyclovir in the treatment of opportunistic HSV-1 infections in LP-BM5 virud-infected mice. Thus, like AZT, PMEA was effective against retrovirus infection, and, like acyclovir, PMEA was effective against

HSV-1 infection. This gives PMEA the unique potential of being useful in the treatment of opportunistic HSV infections as well as the underlying retroviral disease.

# INTRODUCTION

The discovery and development of drugs effective against the human immunodeficiency virus (HIV) or against the opportunistic pathogens commonly associated with AIDS is made difficult by the lack of suitable and convenient animal models. Several conventional murine retrovirus models such as Friend, Rauscher, and Moloney leukemia are currently used to determine the *in vivo* efficacy of new antivirals. While these animal models are useful in measuring the ability of a drug to prevent or suppress retrovirus replication, they tell us little about its ability to prevent or suppress the development of viral-induced immunosuppression and susceptibility to opportunistic infections.

Infection of C57BL/6 mice with the LP-BM5 retrovirus complex causes an AIDS-like disease (MAIDS) the pathology of which at least partially resembles human AIDS (14, 16, 17, 22). Because of the similarities to human AIDS and the ease with which disease manifestations can be measured, the MAIDS model provides a safe and inexpensive means with which to evaluate the *in vivo* efficacy of antiretroviral agents as well as other agents which may be useful in the treatment of opportunistic infections. Moreover, this model could be used to establish a base from which therapeutic strategies to be employed in other more costly feline and primate models could be designed.

A number of 3-hydroxy-2-phosphonylmethoxypropyl (HPMP) and 2-phosphonylmethoxyethyl (PME) derivatives of purine and pyrimidine have been evaluated for their antiviral properties (1, 5, 6, 7, 13). In general, HPMP analogues are more active than PME compounds against vaccinia, adeno-, cytomegalo- and varicella-zoster viruses. In contrast, PME analogues are more effective than HPMP analogues against the human immunodeficiency virus. Nonetheless, both HPMP and PME analogues are equally effective against herpes simplex viruses (HSV).

Because of their broad-spectrum activity against DNA viruses and retroviruses, both HPMP and PME analogues offer a wealth of potential applications, particularly in the treatment of AIDS and the opportunistic infections associated with immunosuppression. Recently, Balzarini et al. (1) demonstrated the *in vivo* antiretroviral activity of one PME analogue, 9-(2-phosphonylmethoxyethyl) adenine (PMEA), while De Clercq et al. (7) demonstrated its *in vivo* anti-HSV activity. This study extends previous *in vivo* observations and further demonstrates the therapeutic potential of PMEA in a retroviral model of immunosuppression and opportunistic HSV-1 infection.

# MATERIALS AND METHODS

## Virus and Animals

LP-BM5 virus was kindly provided by Dr. Robert Yetter, VA Hospital, Baltimore, MD., and was maintained in a persistently infected SC-1 cell line (TC 2110). Working stocks of virus were prepared by overlaying TC 2110 cells with rapidly growing uninfected SC-1 cells maintained in Eagle's MEM supplemented with 5% fetal calf serum, penicillin and streptomycin. These cultures were incubated for 24 hours and supernatant fluids harvested, centrifuged at 1000 x g for 30 minutes and filtered through a 0.22 um membrane filter. Virus stocks were stored at -80$^{o}$C for up to 6 months prior to use. Female C57BL/6 mice (28-33 days old; CIBA-GEIGY, Sisseln, Switzerland) were infected intraperitoneally with 0.5ml of undilute virus. A complete description of the LP-BM5 virus complex and the immunosuppression which occurs following infection has been reported elsewhwere (12, 14-16).

A non-neurovirulent strain (VR3) of herpes simplex type 1 virus was obtained from the laboratory of Dr. A. Nahmias (Emory University, Atlanta, GA.) and propagated in Vero cells. Virus stocks contained 2.5 x $10^{7}$ pfu/ml and were stored at -80$^{o}$. A complete description of this virus and the murine model of pneumonitis which is induced in young (4-week-old) mice following intranasal instillation has been

reported elsewhere (9, 20); older mice are resistant to infection with this virus unless immunosuppressed.

A neurovirulent strain of HSV-1 (McIntyre) was obtained from the American Type Culture Collection (ATCC VR-539) and propagated in human embryonic foreskin cells (7000, Flow Laboratories Inc., McLean, VA). Culture supernatants were harvested at 72 hours after infection, clarified by centrifugation (1000 x g for 20 min.) and stored at -80°C. The virus titer, as assessed on Vero cells, was $1.0 \times 10^8$ pfu/ml.

Drugs

Hybrid recombinant human alpha-interferon (rhuIFNalpha-B/D; CGP 35269) was produced in yeast and purified as previously described (15). This interferon was biologically active in a variety of animal species including mice and was stored carrier-free in PBS at 4°C at a concentration of 0.2mg/ml ($3 \times 10^7$ international units).

Azidothymidine (AZT), was synthesized in the Chemistry Department at CIBA-GEIGY, Basel, Switzerland, and dissolved in distilled water at a concentration of 0.2 mg/ml. This solution replaced distilled water for drinking in those animals receiving AZT. Water consumption for each cage was recorded and the average dose of AZT which each mouse received calculated from this information. Mice receiving AZT consumed approximately 3ml per day.

9-(2-Phosphonomethoxyethyl) adenine (PMEA) was kindly provided by Dr. A. Holy (Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Sciences, Flemingovo namesti 2, 16610 Praha 6, Czechoslovakia). This drug was dissolved in phosphate buffered saline prior to intraperitoneal injection. Acyclovir (Burroughs Wellcome, Research Triangle Park, NC) and Ribavirin (Viratek, Palo Alto, CA) were also dissolved in phosphate-buffered saline prior to use.

Mitogen response of splenocytes to con A

A rapid colorimetric assay for cell proliferation was used to evaluate the response of splenocytes to stimulation by con A. The assay has been descriibed (19) and is based on the ability of the dehydrogenases present in the mitochondria of living cells to cleave the tetrazolium ring of 3-(4,5,dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT). Briefly, $2 \times 10^5$ splenocytes were cultured for 48h in the presence of 4ug of concanavalin A. MTT was then added and the products of cleavage determined colorimetrically on a Dynatech Microelisa reader at a wavelength of 570nm.

Determination of LP-BM5 titers in spleen homogenates.

Virus titers in the supernatants of 10% spleen homogenates were determined by a focal immunoassay based on the method described by Chesebro and Wehrly (ref.3 and personal communication). Briefly, SC-1 cells wre seeded in 24-well tissue culture plates at a density of 4-5 $\times 10^4$ cells per well. DEAE-dextran (10ug/ml) in serum-free medium was added 18 hours later and the monolayers incubated for an additional 30

min.. After washing with serum-free medium, monolayers were infected with virus diluted in RPMI medium containing 0.1% FCS and incubated for two hours. Complete medium containing 10% FCS was added to infected monolayers and the cells incubated at 37°C for 3-4 days. At the end of this incubation period, culture medium was removed and the monolayers incubated with undiluted culture supernatant from a hybridoma secreting rat IgG2a reactive against gp70 of murine leukemia viruses (hybridoma 83A25 kindly provided by Dr. Leonard Evans and Bruce Chesebro, NIH, Rocky Mountain Lab., Hamilton, MT).

Following addition of the primary anti gp70 hybridoma antibody, cell monolayers were washed with PBS+1% FCS and fixed with absolute methanol. Fixed monolayers were incubated with peroxidase-conjugated anti-rat IgG (Sigma) for 45 min. at room temperature and washed three times with Tris buffered saline (pH 7.5) containing 0.002M EDTA and aminoethyl carbazole. A solution containing hydrogen peroxide was then added and plates held in the dark for 30 minutes. Cell monolayers were washed with water, air dried and examined with a dissecting microscope. Foci were counted and the results expressed as focus forming units (ffu) per gram of spleen.

Superinfection with HSV-1 and treatment with antivirals

Mice at 60-80 days post LP-BM5 infection were infected with either HSV-1/McIntyre by intraperitoneal injection of $5 \times 10^5$ pfu per mouse or HSV-1/VR3 by instilling $1 \times 10^5$ pfu into the external nares of ether-anaesthetized mice. Antivirals (acyclovir, ribavirin or PMEA)

were administered intraperitoneally on the day of superinfection and on days 2, 4, and 6 following infection. Deaths were recorded and analyzed by Wilcoxon rank analysis using a one-tailed test. Each treatment group consisted of at least 10 mice.

# RESULTS

## Therapeutic activity of rhuIFNalpha-B/D, PMEA and AZT in the treatment of MAIDS: Effects of early drug intervention.

C57BL/6 mice infected with the LP-BM5 virus complex developed: i) pronounced splenomegaly, ii) mitogen unresponsive splenocytes, iii) detectable levels of infectious virus in spleens, and iv) susceptibility to superinfection with HSV-1. Although treatment with AZT or PMEA beginning on the day of virus infection and continuing for either 21 or 60 days could not prevent these changes from occurring, both drugs slowed the progression of immunosuppression and reduced susceptibility to HSV-1 superinfection (Table 1). Recombinant alpha B/D interferon did not have a demonstrable effect on the immunological parameters studied but did have a modest effect on susceptibility to HSV-1 superinfection.

## Therapeutic activity of PMEA alone or in combination with interferon : Effects on established disease.

Treatment of LP-BM5 virus-infected mice with PMEA or AZT starting 23 days after infection did not result in a significant improvement in either the immunological or virological parameters studied (Table 2). Moreover, mice receiving drug therapy at this late stage of the disease were virtually as susceptible as untreated controls to experimental superinfection with HSV-1. In contrast, mice receiving both PMEA and rhuIFN-alpha B/D had reduced levels of infectious virus

in their spleens and were markedly resistant to HSV-1 infection. Treatment with this combination had no effect on spleen enlargement or mitogenic responsiveness.

Treatment of opportunistic HSV-1 infections in mice immunosuppressed by LP-BM5 virus infection.

LP-BM5 immunosuppressed mice were susceptible to experimental infection with HSV-1 two months after their initial retrovirus infection; whereas, age-matched immunocompetent littermates were fully resistant.

Acyclovir (Figure 1) and PMEA (Figure 2) were equally effective in prolonging the mean survival time of immunosuppressed mice experimentally infected (intraperitoneally) with a neurovirulent strain of HSV-1 (McIntyre) that ultimately disseminated to the brain. Ribavirin was inactive against the neurovirulent HSV-1 infection (Figure 1) as well as pneumonic (VR/3) HSV-1 infections that remained confined to the lung following intranasal instillation. In contrast, PMEA was highly effective in the treatment of the pulmonary HSV-1 infection (Figure 3).

## DISCUSSION

Few animal models exist which are useful for evaluating antiviral agents which may be effective in the treatment of human AIDS. Most of the animal models (primate, bovine or feline) considered relevant are too expensive for routine drug evaluation and too difficult to handle and maintain. The MAIDS model offers the advantage of being inexpensive and easy to handle. The classical AIDS drug, AZT, was quite effective in controlling virus replication and immunosuppression when therapy was initiated on the day of LP-BM5 virus infection. In contrast, AZT was not effective when given to mice with established disease (23 days post-infection). Thus our observations on the therapeutic effects of AZT in MAIDS are similar to those with AZT in human AIDS (4) and in other murine retroviral models (23).

Both *in vitro* and *in vivo* antiretroviral and antiherpesviral activity of PMEA has been reported (1, 5-7). The present study has used the MAIDS model to demonstrate that PMEA was as effective as AZT in inhibiting virus replication and delaying immunosuppression. Like AZT, PMEA was not effective in reversing or slowing the course of established disease.

We previously reported the synergistic response observed when rhuIFNalpha-B/D was used with AZT in the treatment of MAIDS (8). In this study, rhuIFNalpha-B/D used in combination with PMEA was more effective than either drug alone when administered late in the course

of MAIDS. While the mechanism by which rhuIFNalpha-B/D complements PMEA is not clear, it is quite likely that both drugs exert their virustatic effects at different points in the viral replicative pathway. Thus PMEA may interfere with the synthesis of viral nucleic acid while interferon may augment immune surveillance and induce the synthesis of cellular proteins which inhibit viral replication.

As a consequence of the marked immunosuppression caused by infection with LP-BM5 virus, mice develop susceptibility to experimental superinfection with HSV-1 (8) or ectromelia virus (2). This feature has allowed us to determine the efficacy of PMEA against opportunistic infections in immunocompromised mice. Both PMEA and acyclovir afforded mice a significant degree of protection to infection with either the McIntyre or VR3 strain of HSV-1. In contrast, ribavirin was inactive. Thus PMEA has the unique advantage of being useful in the treatment of opportunistic HSV infections as well as the underlying retroviral cause of immunosuppression. This feature of PMEA may be of great value in view of the observation that herpes viruses may act as co-factors in the onset and progression of human AIDS (18).

While a number of disease parameters (e.g. spleen weight, mitogenic response of splenocytes, viral titers, and susceptibility to superinfection) have been used to monitor drug efficacy in the MAIDS model, it is difficult to conclude which parameter is the most relevant. However, the fact that the usual cause of death in AIDS patients results from opportunistic infections suggests that

resistance to superinfection may be a highly significant determinant of drug efficacy. Additional studies which more fully characterize the susceptibility of LP-BM5 virus-infected mice to other opportunistic (bacterial, fungal or protozoan) pathogens are currently in progress.

In conclusion, this study has demonstrated the value of the broad spectrum antiviral agent, PMEA, on both early retroviral replication and opportunistic infections which appear late in the course of immunosuppression. Moreover, our data support the concept that the MAIDS model, in combination with experimentally induced bacterial or viral superinfection, provides a suitable alternative to drug evaluation in more expensive and less convenient animal models of retrovirus-induced immunosuppression.

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The authors wish to thank Mr. Benno Grabshire and Mr. Bernard Poncioni for their technical assistance in this study.

## FIGURE LEGEND

Figure 1. Survival of LP-BM5 virus-infected mice following challenge (65 days post-LP-BM5 virus infection) with a neurotropic strain of HSV-1 (McIntyre) and treatment with acyclovir or ribavirin. Mice were infected intraperitoneally (5 x $10^5$pfu) and treated with acyclovir (200 mg/kg, i.p.) or ribavirin (500 mg/kg, i.p.) beginning on the day of virus infection and again on days 2, 4 and 6 following infection. Immunocompetent controls (o); immunosuppressed controls (no drug) (•); ribavirin treated (■); acyclovir treated (□). Statistical evaluation (10 mice per group) using Wilcoxon rank analysis.

Figure 2. Survival of LP-BM5 virus-infected mice following challenge (75 days post-LP-BM5 virus infection) with HSV-1/McIntyre and treatment with PMEA. Mice were infected intraperitoneally (5 x $10^5$pfu) and treated with 100 mg/kg of PMEA beginning on the day of virus infection and again on days 2,4, and 6 following infection. Immunocompetent controls (o); immunosuppresssed controls (no drug) (•); PMEA treated (□). Statistical evaluation (10 mice per group) using Wilcoxxon rank analysis.

Figure 3. Survival of LP-BM5 virus-infected mice following challenge (75 days post-LP-BM5 virusinfection) with a pneumonic strain (VR3) of HSV-1 and treatment with PMEA. Mice were infected

intranasally with $1 \times 10^5$ pfu and treated with 100 mg/kg of PMEA beginning on the day of virus infection and again on days 2, 4 and 6 following infection. Immunocompetent controls (o); immunosuppressed controls (no drug) (•); PMEA treated (□). Statistical evaluation (10 mice per group) using Wilcoxon rank analysis.

Table 1. Effect of continuous (day 0-59) or early (day 0-22) therapy with PMEA, AZT or recombinant human interferon on the development of immunosuppression in LP-BM5 virus infected mice.

| Treatment | Spleen Weight (mean ± SD) | | Splenocyte Mitogen Response[a] (stimulation index) | | Splenic Virus titer[b] (ffu/g) | | HSV-1 Challenge (% survivors) |
|---|---|---|---|---|---|---|---|
| | t=23[d] | t=60 | t=23 | t=60 | t=23 | t=60 | |
| Non-immunosuppressed | 59 ± 21 | 79 ± 45 | 2.15 | 2.19 | — | — | 100 |
| Immunosuppressed no drug therapy | 198 ± 55 | 520 ± 87 | 1.60 | 1.04 | 1130 | 8120 | 8 |
| AZT[e] 30 mg/kg daily days 0-22 | 87 ± 38 | 383 ± 53 | 1.74 | 1.11 | 615 | 1650 | 60 $p < 0.05$ |
| PMEA 25mg/kg daily days 0-22 | 59 ± 16 | 325 ± 9 | 1.93 | 1.14 | 2390 | 7680 | 80 $p < 0.005$ |
| days 0-59 | 109 ± 13 | 195 ± 95 | 1.97 | 1.47 | 1430 | 1540 | 90 $p < 0.005$ |
| PMEA 5mg/kg daily days 0-22 | 141 ± 55 | 394 ± 81 | 1.79 | 1.02 | 1300 | 4150 | 50 $p < 0.05$ |
| rhuIFN[f] $5 \times 10^7$ IU/kg 3x/weekly days 0-22 | 157 ± 61 | 444 ± 34 | 1.47 | 1.03 | 3820 | 4130 | 40 |

a. Concanavalin A response in pooled splenocytes; three mice per group.

b. Focus forming units (ffu) per gram spleen weight as measured by immunoperoxidase staining.

c. McIntyre strain of HSV-1 ($5 \times 10^5$ pfu, i.p.) 60 days following infection with LP-BM5 virus. Ten mice per group.

d. Time in days after LP-BM5 virus infection.

e. Azidothymidine administered in drinking water (p.o.); 30mg/kg is an average dose based on the observation that mice consume approximately 3ml/day.

f. 9-(2-Phosphonylmethoxyethyl) adenine dissolved in PBS and administered intraperitoneally.

g. Recombinant human interferon alpha B/D hybrid dissolved in PBS and administered subcutaneously.

Table 2. Effect of delayed drug therapy with PMEA, AZT and /or recombinant human interferon on the development of immunosuppression in LP-BM5 virus infected mice.

| Treatment | Spleen Weight (mean ± SD) | | Splenocyte Mitogen Response[a] (stimulation index) | | Splenic Virus titer[b] (ffu/g) | | HSV-1 Challenge (% survivors) |
|---|---|---|---|---|---|---|---|
| | t=23[d] | t=60 | t=23 | t=60 | t=23 | t=60 | |
| Non-immunosuppressed | 59 ± 21 | 79 ± 45 | 2.15 | 2.19 | — | — | 100 |
| Immunosuppressed Cont. (no drug therapy) | 198 ± 55 | 520 ± 87 | 1.60 | 1.04 | 1130 | 8120 | 8 |
| AZT[e] 30mg/kg daily days 23 - 59 | 167 ± 35 | 490 ± 42 | 1.65 | 1.05 | 1660 | 1140 | 11 |
| PMEA 25mg/kg daily days 23 - 59 | 236 ± 33 | 587 ± 111 | 1.58 | 0.97 | 2400 | 4090 | 20 |
| rhuIFN[g] - B/D $5 \times 10^7$ u/kg 3x/week days 23 - 59 | 222 ± 20 | 602 ± 172 | 1.31 | 0.94 | 3160 | 2310 | 0 |
| PMEA 25mg/kg daily + $5 \times 10^7$ IU/kg rhuIFN 3x/week days 23 - 59 | 169 ± 42 | 431 ± 147 | 1.72 | 1.04 | 3750 | 456 | 70 $p \leq 0.005$ |

a. Concanavalin A response in pooled splenocytes; three mice per group.

b. Focus forming units (ffu) per gram spleen weight as measured by immunoperoxidase staining.

c. McIntyre strain of HSV-1, ($5 \times 10^5$ pfu, i.p.) 60 days following infection with LP-BM5 virus. Ten mice per group.

d. Time in days after LP-BM5 virus infection.

e. Azidothymidine administered in drinking water (p.o.); 30mg/kg is an average dose based on the observation that mice consume approximately 3ml/day.

f. 9-(2-Phosphonylmethoxyethyl) adenine dissolved in PBS and administered intraperitoneally.

g. Recombinant human interferon alpha B/D hybrid dissolved in PBS and administered subcutaneously.

Figure 1

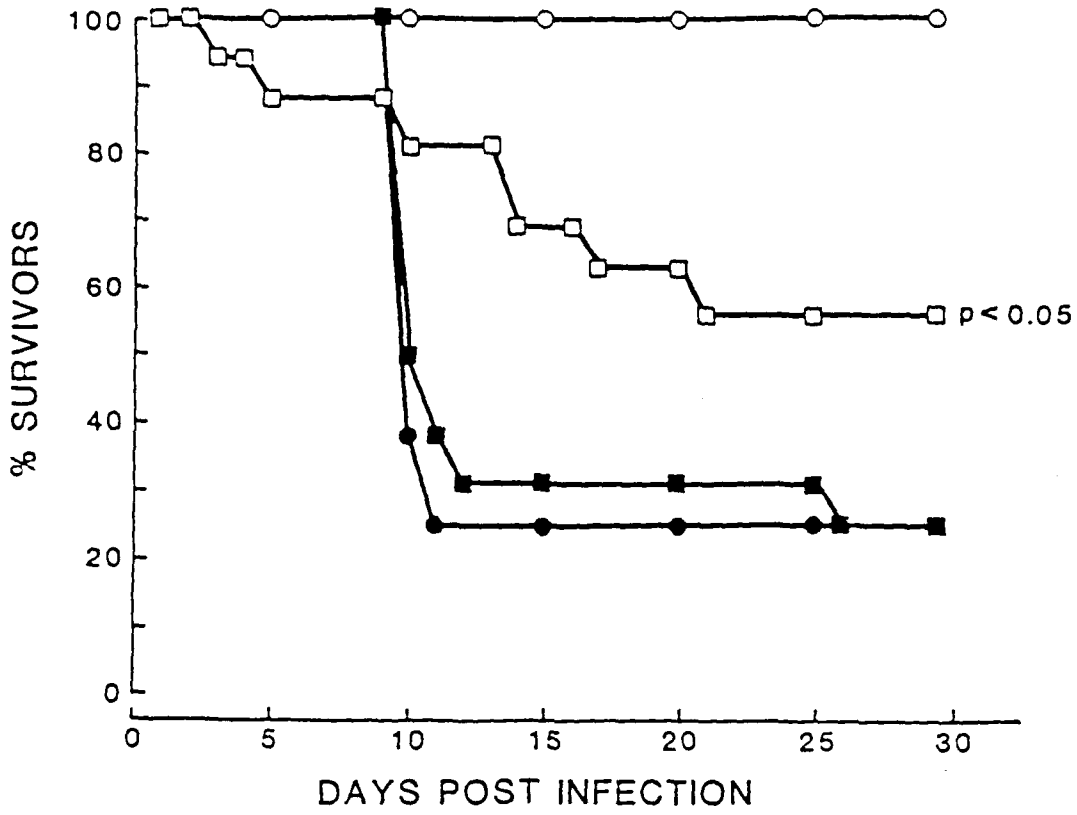
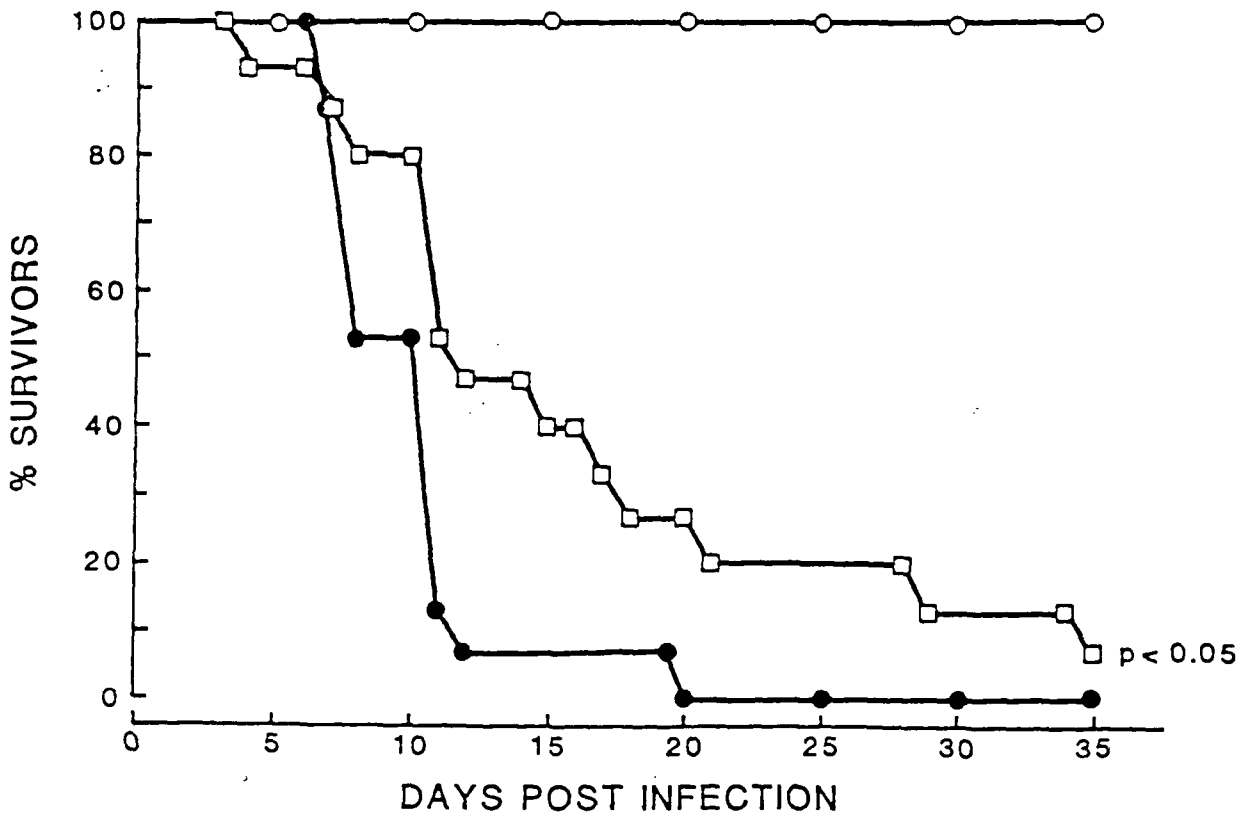

100
80
60
40
20
0
% SURVIVORS
0
5
10
15
20
25
30
DAYS POST INFECTION
p < 0.05

Figure 2


100
80
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% SURVIVORS
0
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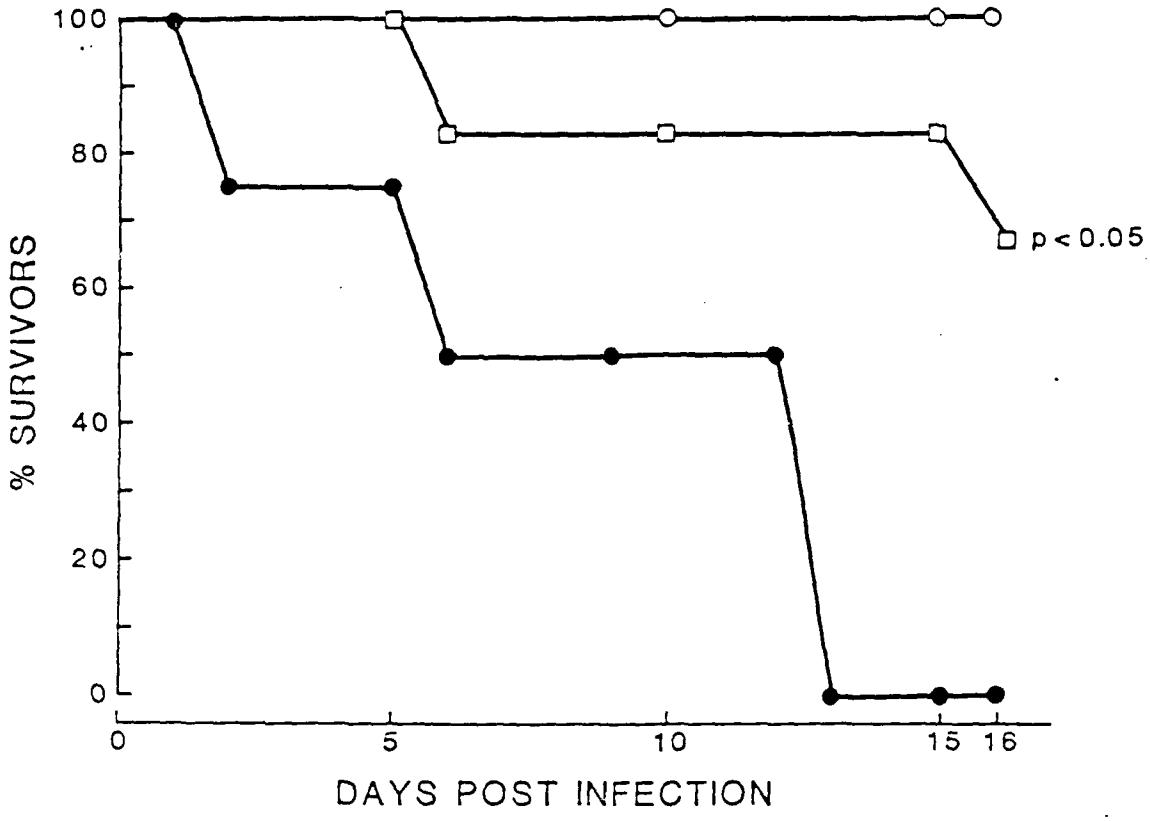
35
DAYS POST INFECTION
p < 0.05

Figure 3


100
80
60
40
20
0
% SURVIVORS
0
5
10
15
16
DAYS POST INFECTION
p < 0.05

APPENDIX II

# USE OF HUMAN MONOCYTES IN THE EVALUATION OF ANTIVIRAL DRUGS: QUANTITATION OF HSV-1 CYTOPATHIC EFFECTS

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Key words: Human monocytes, differentiation, herpes simplex type 1 virus, alpha interferon, poly I:C-LC and Acyclovir, viability index, drug screening.

Running title: Human Monocytes in the Evaluation of Antiviral Drugs.

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## SUMMARY

An assay for the evaluation of antiviral and immunomodulator potency was developed using pure populations of cultured human monocytes. The assay involved culturing of human monocytes until they were fully susceptible (15-20 days) to lytic infection with HSV-1. When susceptible cells were cultured for 12-18 hours with recombinant interferon alpha or the interferon inducer poly I:C-LC, a significant enhancement in resistance to the cytopathic effects of HSV-1 was observed. Similarly, a dose dependent reduction of cytopathology was observed when acyclovir or ribavirin were added immediately after cell infection. Resistance to HSV-1 induced cytopathology was determined by neutral red dye uptake and was quantified by a colorimetric procedure. The antiviral assay described is highly sensitive and reproducible.

# INTRODUCTION

A number of assay systems using either plaque reduction or cytopathic effect measurements and employing a variety of cell types are currently being used to establish the potency of antiviral agents. Unfortunately, the data generated in these assays are derived from cells (e.g. Vero, primary rabbit kidney, human embryonic skin or muscle, mouse embryo) which are irrelevent to the pathology that the virus induces in man. In contrast, cells of the mononuclear phagocyte system are of greater relevance for the determination of antiviral efficacy since these cells constitute the first line of cellular defense against most viruses (Morahan et al., 1985).

The use of mononuclear phagocytes as a primary culture system for the evaluation of antiviral agents has been hampered due to i) inadequate cell yield, ii) contamination with other cell types, and iii) lack of adequate long-term maintenance procedures. We have been successful in overcomming these difficulties and have established a methodology for the production of homogeneous populations of *in vitro* differentiated human blood monocytes. These cells show a profound increase in permissiveness to HSV-1 replication and result in a rapid and dramatic cytopathic effect which can be quantitated using a colorimetric procedure.

The value of the human mononuclear phagocyte antiviral assay described in this study was documented using several well- known antiviral agents including acyclovir, ribavirin, poly I:C-LC and alpha interferon. Our results indicate that this system provides a suitable

model for the evaluation of antiviral and immunomodulating agents in a highly relevant cell.

# MATERIALS AND METHODS

## Preparation of human mononuclear cells

Mononuclear cell enriched leukocyte concentrates were obtained from normal donors undergoing lymphocytapheresis with the help of a Fenwal C.S. 3000 continuous flow centrifugation unit (Stevenson et al.,1983). Two hundred milliliters of concentrate containing approximately 7.5 x $10^9$ leukocytes were diluted 1:4 with sterile PBS (Dulbecco's w/o calcium and magnesium) and 35 ml layered over 15 ml of Lymphoprep™. (Nycomed A/S Oslo, Norway). After centrifugation for 40 minutes at 23° C and 1200 rpm (600 x g), the mononuclear cells were collected from the interphase and washed twice in elutriation media (Hanks' Balanced Salt Solution, w/o calcium and magnesium containing 2% human serum albumin [Bern Blood Bank]). Cells were suspended in elutriation medium and 250 ml (3 x $10^9$ cells) loaded into the seperation chamber of a Beckman JE-6B elutriation system which was placed in a Beckman J2-21M induction drive centrifuge. Sample loading was done at 5°C at a flow rate of 14.6 ml/min. and a rotor speed of 2500 rpm . Three fractions were collected. Fraction 1 consisted of the first 500 ml of eluate and fraction 2 consisted of the next 150 ml collected at a flow rate of 15.4 ml/min. Fraction 3 (200 ml) was collected at a flow rate of 25 ml/min. Each fraction was centrifuged at 600 x g for 10 minutes and washed twice in RPMI-1640 containing 1% human A/B serum. Cell purity was assesed following cytocentrifugation (Cytospin 2, Shandon Instruments, Sewickley, PA). Morphological criteria together with

cytochemical staining were used to determine monocyte purity. Fraction 3 contained greater than 97% monocytes, and less than 1% lymphocytes and neutrophils, and was used in these studies.

Virus preparation

Herpes simplex type 1 virus (strain VR/3) was obtained from the laboratory of Dr. A. Nahmias (Emory University, Atlanta, GA.) and passaged in Vero cells grown in RPMI medium containing 5% fetal calf serum, 100 units/ml penicillin and 100 ug/ml streptomycin. Virus stocks were titrated on Vero cells and contained $7.5 \times 10^7$ pfu/ml. Virus was stored in 1ml ampoules and frozen at -80°C until used.

In vitro culture of monocytes

Monocytes were allowed to differentiate in "Sterilin" bacteriological petri dishes in RPMI-1640 containing 10% human AB serum, 50 units/ml penicillin, 50 ug/ml streptomycin, 2mM L-glutamine and 1mM sodium pyruvate (complete RPMI). Ten million cells suspended in 10 ml were cultured in each plate and detached at various times by washing with cold PBS (w/o calcium and magnesium) prior to adding to microtiter plates and infection with HSV-1.

Virus Infection and Addition of Drugs

Differentiated cells ($3 \times 10^4$) suspended in a volume of 0.2 ml of complete RPMI were added to each well (except for peripheral wells) of a 96-well microtiter plate and incubated in the presence of optimal and suboptimal concentrations of poly I:C-LC or recombinant human

interferon-alpha for 18-24 hours prior to infection. These cells were either mock infected (RPMI without serum) or infected with 0.025 ml of the VR/3 strain of HSV-1 at a multiplicity of 0.5, 1.0 and 2.0 plaque forming units per cell. Virus was adsorbed at 37$^{o}$ C (rocking plates every 15 minutes) for 1 hour after which 0.2 ml of complete RPMI medium was added. When testing nucleoside analogues such as acyclovir or ribavirin, which do not require a preincubation period, drug was added in complete RPMI to each well following the infection procedure described above.

Evaluation of HSV-1 Cytopathic Effects

Cell viability following virus infection was measured by removing the media from infected cells and adding 0.2 ml of a filtered (0.22 u), neutral red (0.01% final concentration in RPMI) solution containing 1% human serum and 25 millimolar HEPES. Plates were incubated for one hour at 37 $^{o}$ C and the neutral red solution removed. Sorenson's citrate ethanol buffer (pH 4.1) in a volume of 0.1 ml was added to extract cell associated dye and the resulting color measured in a colorimeter at 540 nm. The viability index was expressed as the ratio of dye uptake by infected cells to dye uptake by uninfected cells.

Drugs

Recombinant human IFN alpha (rhuIFN-alpha) was obtained from Hoffmann-La Roche and stored carrier free in PBS at 4 degrees C at a

concentration of 1 x $10^6$ units/ml and was diluted in RPMI prior to use.

Poly inosinic: poly cytodylic acid conjugated to lysine carboxymethylcellulose (poly I:C-LC) was obtained from Dr. Hilton Levy (NIH, Wash. DC) at a concentration of 1 mg/ml and was diluted in RPMI.

Acyclovir was a generous gift from Burrough's Wellcome Laboratories, Research Triangle Park, NC and diluted in complete RPMI prior to adding to infected cells. Ribavirin was obtained from the Antiviral Studies Group, USAMRIID, Fort Detrick, Frederick, MD.

## RESULTS

### Quantitation of HSV-1 Cytopathic Effects

Monocytes kept in culture for two weeks increased in size (10 x), remained esterase positive and became peroxidase negative, displayed high pinocytic and phagocytic activity, and produced IL-1 when stimulated with LPS (data not shown). When these cells were infected with HSV-1, the following morphological changes were observed: 1) cell rounding was observed by 8 hours and fusion by 12-16 hours post-infection, 2) fusion progressed to syncytial formation by 24-48 hours and was temporally related to the input m.o.i. and cell density, and 3) cell lysis and death correlated with a reduction in neutral red uptake and was complete 48-72 hours after infection. Figure 1 illustrates the relationship between intensity of cell lysis, m.o.i., and time post infection.

## Human Monocyte Susceptibility to HSV-1 Lysis: Dependence on in vitro Differentiation

Cells were cultured for various times, removed from culture dishes, added into 96 well plates and infected with HSV-1. As illustrated in Figure 2, fresh monocytes were not susceptible to lytic infection, but became susceptible following prolonged time in culture. Susceptibility to virus lysis was acquired at a time when these cells developed classical macrophage morphology.

## Correlation Between Observed Cytopathic Effects and Release of Infectious Virus

To determine the extent of infectious virus released by cultured monocytes, plaque titrations were performed on cell supernatants at selected times following infection. As shown in Table 1, release of infectious virus was observed at 24 hours post infection and remained elevated up to the time of cell lysis (72 hours).

## Modulation of HSV-1 Induced Cytopathic Effects by Selected Pharmacological Agents

Experiments to determine antiviral activity of immunomodulators and nucleoside analogues were designed. Figure 3 illustrates the effect of incubating two-week old monocytes with poly I:C-LC (3a) or rhuIFN-alpha (3b) for 18 hours prior to virus infection. The addition of poly I:C-LC resulted in a dose dependent response. Inhibition of HSV-1 cytopathology was observed at concentrations ranging between 3

and 27ug/ml with an $ED_{50}$ of approximately 6ug/ml. independent of the m.o.i. examined. Likewise, alpha interferon exhibited a dose dependent response and inhibition of viral cytopathology was observed between 10 and 100 units/ml; however, the degree of protection was dependent on the m.o.i..

In contrast to immunomodulators, the presence of nucleoside analogues was required throughout the virus incubation period. Ribavirin (Figure 3c) was only marginally effective in this assay system when examined at low m.o.i. (0.5). This activity was absent at higher m.o.i.. Higher concentrations of ribavirin (>300 ug/ml) were toxic to target cells. In contrast to ribavirin, acyclovir (Figure 3d) was effective at concentrations ranging from 1-100 nanograms/ml with an approximate $ED_{50}$ of 6 nanograms per ml independent of the m.o.i. examined.

# DISCUSSION

The discovery of novel antiviral drugs should be based on systems that take into account: a) the human origin of the cell target, b) the relevance of the cell to viral pathology, and c) the different mechanisms of action of antiviral agents (virustatic vs immunomodulatory). Ideally such a system could be provided by mononuclear phagocytes which are primary targets for a number of viral infections including HSV-1, HIV and other lentiviruses (Daniels et al., 1978, Koening et al., 1986, Gendelman et al., 1985).

While abortive infections have been observed in human blood monocytes infected with HSV-1 (Daniels et al.,1978), productive HSV-1 infections appear to require cell differentiation (Tenney et al., 1987). Since abortive infections are difficult to quantitate, the development of murine systems or the use of differentiated human macrophage cell lines which are susceptible to productive infection could be used for the discovery of novel antiviral agents. However, these cell systems have limitations such as species specificity for interferons in the case of murine cells or need for chemical agents that are required to induce differentiation and which may alter the effects of the antiviral agents being tested (Tenney and Morahan, 1987).

In this report we have described a cellular system which is based upon the use of spontaneously differentiated human monocytes which are

susceptible to productive HSV-1 infection. We have shown that this productive infection can be abrogated by agents such as interferons, inducers of interferon and nucleoside analogues. Based upon this observation, an assay to evaluate antiviral agents was designed. This assay takes into account both the intensity of infection (moi) and the dosage of drug which are important variables in the determination of potency and efficacy. For example, acyclovir or poly I:C-LC show high potency as well as efficacy, while alpha interferon and ribavirin show efficacy but not potency as indicated by the 50% inhibitory concentrations at three different m.o.i..

The human monocyte system described in this study appears to be more sensitive to antiviral agents. Thus, we were able to show an $ED_{50}$ of 6 nanograms/ml for acyclovir in our system while others have reported values ranging from 200 ng/ml in the case of primary rabbit kidney cells to 10 ng/ml in murine embryonic cells (De Clercq et al., 1986). Furthermore, when bromovinyl-deoxyuridine (BVDU) and hydroxy-2-phosphonyl-methoxypropyl adenine (s-HPMPA) were examined in this cellular system and compared to values reported in the literature (De Clercq et al., 1987), a 10 to 100 fold respective difference was observed. However, the order of potency remained the same (ie. BVDU>acyclovir> (s)-HPMPA>ribavirin) (manuscript in preparation).

In conclusion, we believe that the monocyte/macrophage assay described in this study offers a suitable and relevent model for the evaluation and discovery of novel antiviral agents. This system could

prove useful in the evaluation of drugs which may be effective in the treatment of other macrophage tropic viruses such as CMV and HIV.

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TABLE 1

HSV-1 Replication in Differentiated Human Monocytes

| Hours after infection | p.f.u/ml* | Cell Morphology | Thermal inactivation of virus in culture w/o cells (p.f.u./ml) |
|---|---|---|---|
| 24 | $4 \times 10^6$ | Synctitia | $7 \times 10^5$ |
| 48 | $3 \times 10^6$ | Cell lysis | $6 \times 10^4$ |
| 72 | $6 \times 10^6$ | Total cell lysis | $2 \times 10^4$ |
| 96 | $4 \times 10^6$ | Total cell lysis | $2 \times 10^3$ |

Ten day old cultured monocytes ($2 \times 10^5$ cells) were added to 24 well tissue culture plates and infected with HSV-1 at a multiplicity of 3 and the supernatant fluids collected and titrated (Gangemi et al. 1987) at the times indicated.

*p.f.u. = plaque forming unit

## FIGURE LEGEND

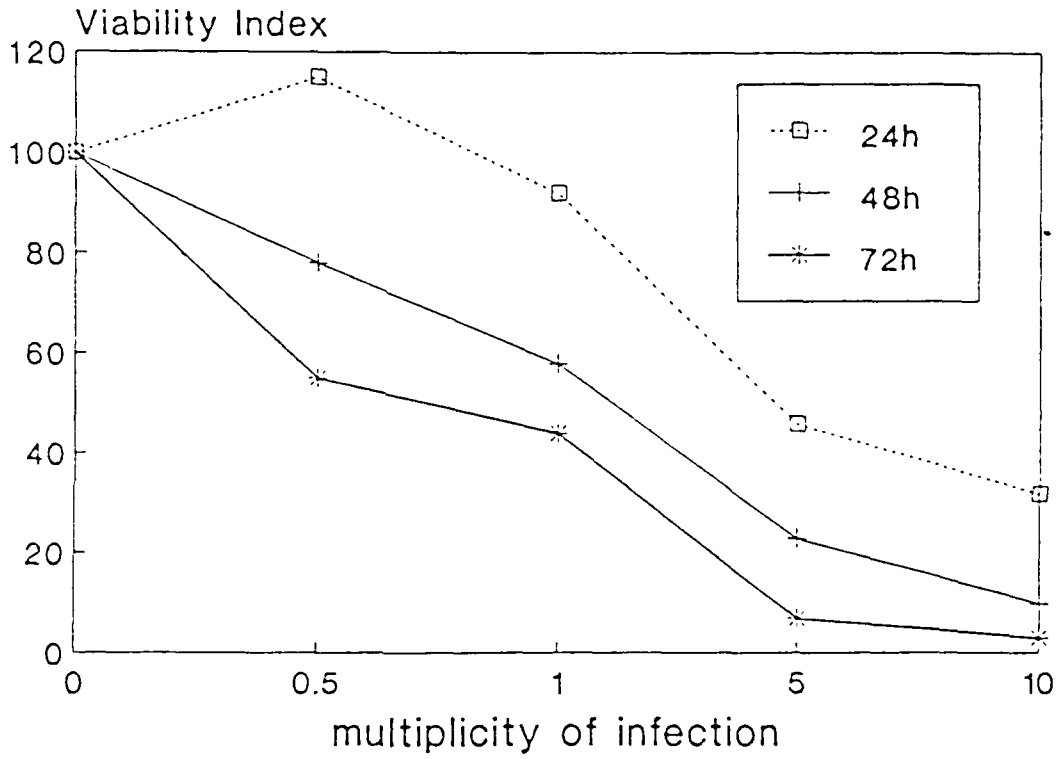
Figure 1: Progression of the cytopathic effect of HSV-1 on *in vitro* differentiated monocytes: use of the neutral red dye uptake for quantification. Two week old macrophages were plated at 3 x $10^4$ cells per well (0.2 ml) adhered overnight, infected as described with an m.o.i. of 0.5, 1, 5 and 10. Neutral red dye uptake was evaluated 24, 48 and 72 hours post infection. Viability index was established based on the following formula:

$$\frac{\text{OD 540 infected cells}}{\text{OD 540 uninfected cells}} \times 100$$

Figure 2: Human monocyte susceptibility to HSV-1 induced cytopathic effects: dependence on *in vitro* differentiation cells from the same donor were cultured for 4, 11, 18 days detached and cryopreserved as described by Stevenson et al. 1983. (Instead of fetal calf serum, human serum was used.) These cells together with the cryopreserved monocytes were thawed, plated to establish confluent monolayer ($3x10^5$ cells/well for monocytes, $10^5$ cells/well for 4 day old cells and 3 x $10^4$ cells/well for 11 and 18 day old cells). After overnight adherence the cells were infected with an m.o.i. of 2. The viability index was determined 72 hours after infection.

Figure 3: Modulation of HSV-1 induced cytopathic effects by selected pharmacological agents. Monolayers of two week old macrophages were established as described. The cells were co-cultured over night with PolyI:C-LC (1, 3, 9 and 30 ug/ml) or IFN-a (10,50,100,500 u/ml) supernatants aspirated and infected at m.o.i. of 0.5 -▲-, 1-■-, 2-●-. When antivirals (Acyclovir 0.3, 1, 3, 10, 30, and 100 ng/ml and Ribavirin 5, 15, 45 and 150 ug/ml) were tested the drugs were added immediately following infection. Seventy two hours later viability index for each drug concentration was determined and ED50 established.

Figure 1


Viability Index
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100
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60
40
20
0
0
0.5
1
5
10
multiplicity of infection
24h
48h
72h

Figure 2

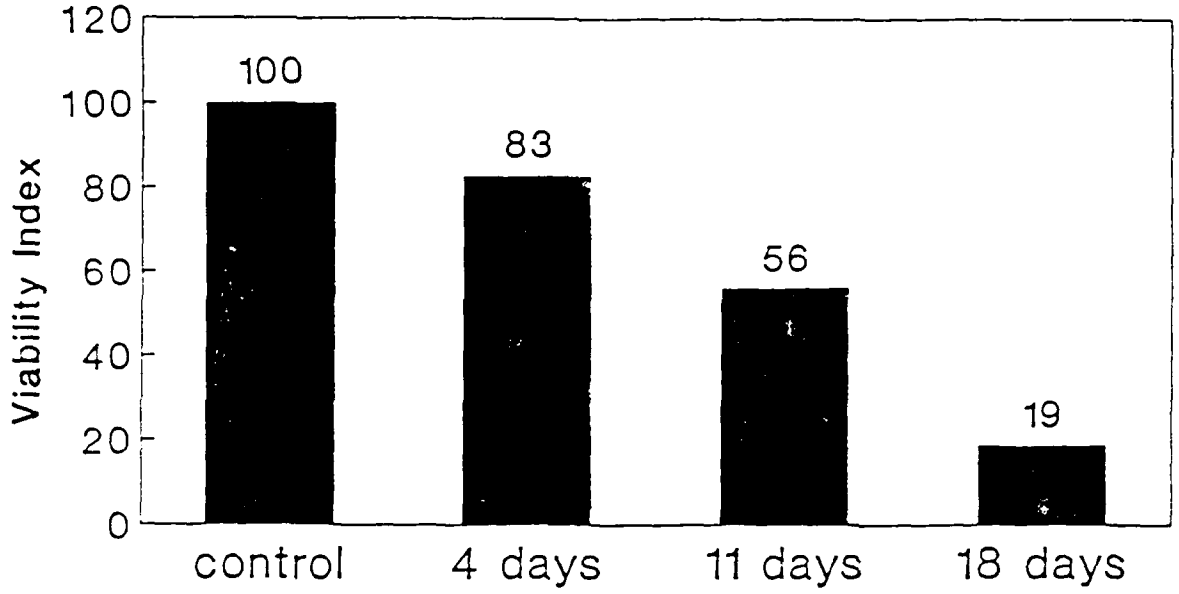
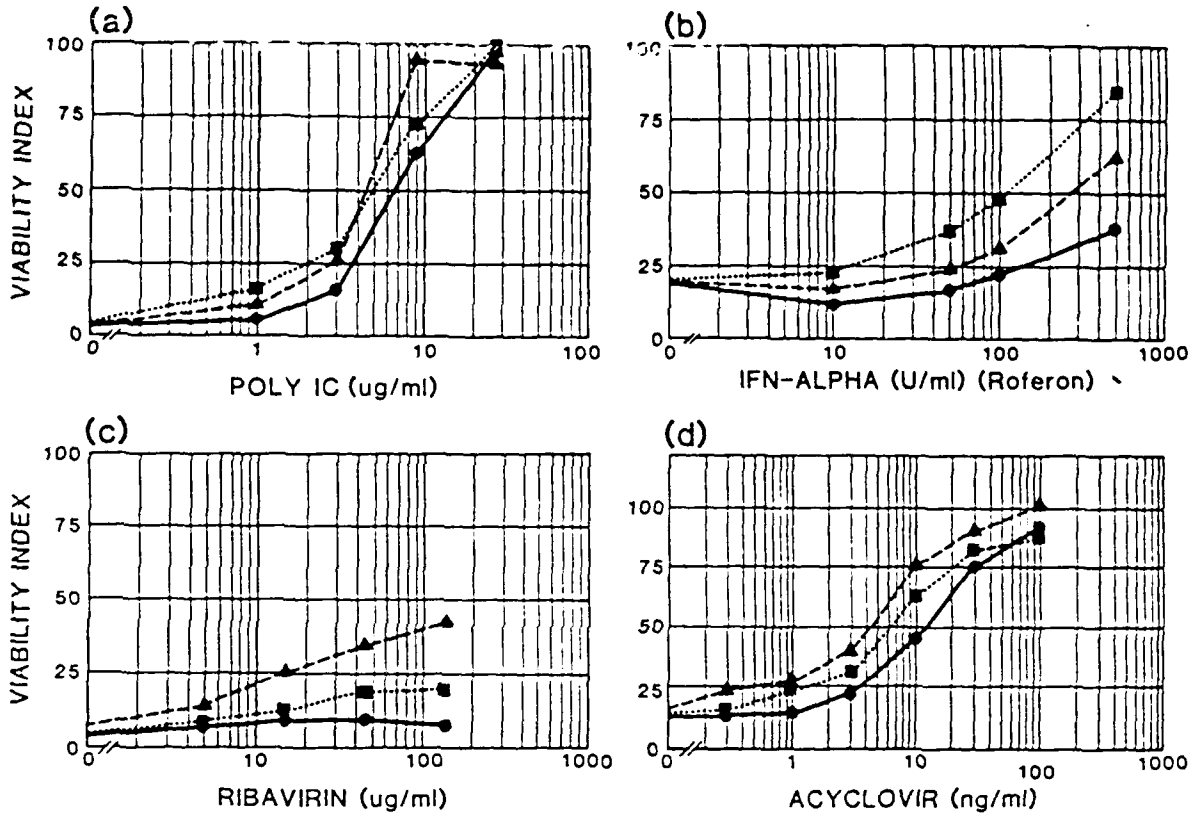

120
100
80
60
40
20
0
Viability Index
100
83
56
19
control
4 days
11 days
18 days

Figure 3


(a)
VIABILITY INDEX
100
75
50
25
0
0
1
10
100
POLY IC (ug/ml)
(b)
75
50
25
0
0
10
100
1000
IFN-ALPHA (U/ml) (Roferon)
(c)
VIABILITY INDEX
100
75
50
25
0
0
10
100
1000
RIBAVIRIN (ug/ml)
(d)
100
75
50
25
0
0
1
10
100
1000
ACYCLOVIR (ng/ml)

APPENDIX III

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APPENDIX III

# Antiviral Activity of a Novel Recombinant Human Interferon-αB/D Hybrid

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## ABSTRACT

**The antiviral potential of a novel cross-species active, recombinant human interferon-αB/D hybrid (rHuIFN-αB/D), was evaluated for its efficiacy in cultured human monocytes and in several murine models of viral disease. When examined in 14-day-old human monocyte cultures, rHuIFN-αB/D was highly effective in preventing viral replication and cell destruction caused by herpes simplex virus type 1 (HSV-1/VR3). The effect observed with 100 units of this hybrid IFN was as good or higher than that observed with equivalent amounts of rHuIFN-αA or IFN-γ. In addition, a single dose ($5 \times 10^7$ U/kg) of rHuIFN-αB/D administered several hours after intranasal infection with HSV-1/VR3 suppressed pulmonary virus replication and prevented death due to interstitial pneumonia. Similarly, mice infected with a more aggressive strain of HSV-1 (McIntyre) were protected when this IFN preparation was administered at the time of virus infection and 1 day later. The anti-retroviral activity of rHuIFN-αB/D was examined in two murine leukemia retroviral models, Rauscher (RMLV) and Friend (FMLV), and a murine model of acquired immunodeficiency (LP-BM5). Treatment of RMLV or FMLV infected mice significantly prolonged mean survival times and the number of long-term FMLV survivors. These therapeutic effects were demonstrated when IFN was administered on the day of virus infection or as late as 3 days following infection. Transient reversal of the immunosuppressive effects induced by LP-BM5 infection was observed when rHuIFN-αB/D treatment was initiated at the time of virus infection. Moreover, when rHuIFN-αB/D was used together with azidothymidine (AZT), the effect of the combination was better than either drug alone.**

## INTRODUCTION

HUMAN INTERFERON-α (IFN-α) consists of many species that are encoded by individual members of the IFN-α multigene family. A few of these species have been expressed in both eukaryotic and prokaryotic cells, and extensive sequence homologies of the genes that encode them have led to

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the construction of hybrid recombinant molecules.[1] One such B/D hybrid, rHuIFN-αB/D (CGP 35 269), consists of amino acids 1-60 and 93-166 from HuIFN-αB and amino acids 61-92 from HuIFN-αD.[2] This hybrid has a broad host-range of antiviral and antiproliferative activity *in vitro*,[3] but has not yet been extensively evaluated *in vivo*.

The currently available recombinant forms of IFN-αA (Roferon-A) and IFN-$\alpha_{2b}$ (Intron A) appear to be therapeutically effective in several human viral infections including AIDS.[4,5] However, these IFNs-α possess undesirable side effects including fever, chills, myalgia, effects on the CNS, and leukopenia when used at dosages required to maintain therapeutic levels; thus, interest in alpha hybrids which may have superior biological activity and reduced toxicity when used over several years or months, has intensified. Since the clinical effectiveness of IFN-α in AIDS possibly includes direct antiviral as well as immunomodulatory and antiproliferative functions, it will be desirable to screen each of these functions independently both *in vitro* and *in vivo* prior to the clinical evaluation of a new hybrid. Moreover, since the antiviral activity of IFN-α hybrids differ when examined against lytic and nonlytic viruses,[6] it will also be desirable to screen new hybrids against infections induced by both. Because of its unique cross-species activity, we have been able to evaluate the antiviral efficacy of rHuIFN-αB/D in mice infected with viruses that cause disease by different pathogenic mechanisms. This study examines the antiviral activity of this hybrid IFN in two murine leukemia models,[7,8] a murine model of AIDS,[9] and a murine model of HSV-1 infection.[10,11]

## MATERIALS AND METHODS

*Virus and Animals:* Working stocks of Rauscher murine leukemia virus, strain 4Q (NIH), were prepared from 10% splenic homogenates. Four weeks after infection, enlarged spleens were homogenized in RPMI medium containing 10% glycerol, centrifuged for 20 min at 1,000 × *g*, the supernatant filtered through a 0.22μ filter, and stored in liquid nitrogen. For experiments, female BALB/c mice (28-33 days old) were infected i.p. with 0.2 ml containing 10 $LD_{50}$ of virus.

Friend virus, polycythemic strain, was obtained from Dr. Michael Dewey (Biology Department, University of South Carolina, Columbia, SC). Working stocks of virus were prepared as described above, except that enlarged spleens from DBA/2 mice were collected 2 weeks after infection. Female, DBA/2, 28- to 33-day-old mice were used in the virus challenge experiments in which 10 $LD_{50}$ of virus in a volume of 0.2 ml was administered i.p.

LP-BM5 virus was obtained from Dr. Robert Yetter (VA Hospital, Baltimore, MD) and was maintained in a persistently infected SC-1 cell line (TC 2120). Working stocks of virus were prepared by overlaying infected cells with uninfected SC-1 cells maintained in minimal essential medium (MEM) containing 5% fetal calf serum and antibiotics. These cultures were incubated for 24-48 h and supernatant fluids harvested, centrifuged at 1,000 × *g* for 30 min, and filtered through a 0.22μ filter. Virus stocks were stored at −80°C prior to use. Female C57BL/6 mice (28-33 days old) were infected experimentally with 0.5 ml (i.p.) of undilute virus. A complete description of this virus and the acquired immunodeficiency disease which occurs in infected mice has been previously published.[9,12,13]

Herpes simplex type-1 virus (strain VR3) was obtained from the laboratory of Dr. A. Nahmias (Emory University, Atlanta, GA) and passaged in Vero cells. Virus stocks were titrated on Vero cells and contained 7.5 × $10^7$ pfu/ml. A complete description of this virus and the pneumonitis which is induced following intranasal instillation in 4-week-old C3H/HeN mice has been previously described.[10,11] HSV-1 McIntyre (ATCC VR-539) was propagated for 72 h in human embryonic foreskin cells (7000, Flow Laboratories Inc., McLean, VA). Culture supernatants harvested after freezing and thawing were cleared by centrifugation and stored at −80°C. Virus titer, as assessed in Vero cells, was 1 × $10^7$ pfu/ml. Dilutions of virus were prepared in Hanks' BSS supplemented with 0.2% bovine serum albumin. Cells, media, and diluents were purchased from GIBCO (Paisley, Scotland). Male BALB/c mice aged 31-33 days and weighing 16.5 ± [illegible] grams on the day of infection (day 0) received i.p. 0.2 ml of diluted virus.

C3H/HeN mice were obtained from Harlan Ltd. (England). DBA/2 mice were obtained from ICO (France) and C57BL/6 and BALB/c mice were obtained from CIBA-GEIBY (Sissel, Switzerland) and were randomly screened for the presence of adventitious agents prior to delivery.

*IFNs and AZT:* The recombinant human IFN-αB/D hybrid (rHuIFN-αB/D), also designated CGP 35 269, was fermented in yeast and purified as described previously.[24] The IFN preparation used in this study was stored, carrier free, in PBS at 4°C at a concentration of 0.2 mg/ml ($3.5 \times 10^7$ units). All dilutions were made in sterile, endotoxin-free PBS (for *in vivo* experiments) or RPMI prior to use. rHuIFN-γ was a generous gift from Kyowa Hakko Kogyo Co., Ltd. (Tokyo, Japan), in sterile vials containing $10^6$ JRU/ml. IFN-αA (Roferon-A) was purchased from Hoffmann-La Roche, Inc. (Nutley, NJ), in vials containing $1 \times 10^6$ U/ml.

Azidothymidine (AZT) was synthesized by CIBA-GEIGY (Basel, Switzerland), and dissolved in water for administration to mice. Mice received either 0.2 or 0.02 mg/ml in their water bottles for 14 or 21 days following infection.

*HSV-1 Infection of Cultured Human Monocytes:* Blood obtained from leukopheresis was used as a source for the isolation of monocytes. White blood cells were collected from the interface of Lymphoprep gradients following centrifugation at 500 × *g* for 40 min, and monocytes recovered by counterflow centrifugation using the Beckman JE-6B rotor (2,500 rpm at 4°C) and elutriation system. This procedure routinely gave monocyte yields that were greater than 95% pure as assessed by cytochemical staining. Monocytes were kept in culture for 10–14 days in RPMI-1640 containing 10% human AB serum, penicillin, streptomycin, L-glutamine, and pyruvic acid prior to plating in 96-well microtiter plates and infection with HSV-1. Forty thousand cells were added to each well, allowed to adhere, and then incubated with rHuIFN-α or -γ at 10, 100, and 1,000 U/ml for 18 h. Treated cells were washed 1 ×, infected with 0.025 ml of HSV-1 at a multiplicity of 0.5, 1, or 2, incubated for 1 h at 37°C, and then refed with 200 μl of complete RPMI medium. Viral induced cytopathology was quantitated by measuring vital dye uptake by monocytes 48 h after infection. Neutral red at a final concentration of 0.02% was added to cell supernatants for 1 h and the cells then washed with PBS. The remaining cell-associated neutral red was extracted in Sorenson's citrate ethanol buffer (pH 4.1) and absorbance read in a colorimeter at 540 nm. The cytopathic index was expressed as percent dye uptake by infected cells compared to dye uptake by uninfected cells.

*Mitogen Response of Splenocytes to Con A:* A rapid colorimetric assay for cell proliferation was used to evaluate splenocyte response to mitogen stimulation. The assay has been previously described and is based on cleavage of the tetrazolium ring of the tetrazolium salt MTT [3-(4,5,dimethyl-thiazol-2yl)-2,5-diphenyl tetrazolium bromide] by dehydrogenases which are active in mitochondria of living cells.[25] Briefly, $2 \times 10^5$ splenocytes are cultured for 48 h in the presence of 4 μg of concanavalin A. MTT is then added and colorimetric measurement of the cleaved dark blue formazan products recorded. Plates were read on a Dynatech microelisa reader using a test wavelength of 570 nm.

*Reverse Transcriptase Assay:* Reverse transcriptase levels in supernatant fluids from 10% splenic homogenates were examined using the procedure of Roy-Burman *et al.*[26] Reference reverse transcriptase was purchased from Bethesda Research Laboratories Life Technologies, Inc. (Gaithersburg, MD).

*Statistical Evaluation:* Statistical evaluation of data was performed using either the Student's *t* test or Wilcoxon rank analysis.

## RESULTS

### *Evaluation of rHuIFN-αB/D in cultured human monocytes infected with HSV-1*

Since macrophages play a critical role in resistance to many viral infections including herpesviruses (and perhaps also to HIV infection), we compared rHuIFN-αB/D with two reference IFNs

(rHuIFN-αA and rHuIFN-γ) for their ability to inhibit lysis of human monocytes following infection with HSV-1. As previously reported,[14] freshly cultured monocytes were resistant to lytic infection with herpesviruses; however, monocytes that were cultured for several weeks prior to infection were highly susceptible to infection (data not presented). As illustrated in Fig. 1, rHuIFN-αB/D was more effective than either rHuIFN-αA or rHuIFN-γ interferon in protecting human monocytes from HSV lysis. This distinction in antiviral potency was evident at each of the three multiplicities of infection (moi) examined. Moreover, rHuIFN-γ concentrations as high as 1,000 units were no better than rHuIFN-αB/D at 100 units (data not presented).

## *Activity of rHuIFN-αB/D in murine models of HSV-1 infection*

rHuIFN-αB/D was effective in the treatment of viral-induced pneumonitis. Greater than 80% protection occurred when a single injection of IFN ($5 \times 10^7$ U/kg) was administrered to mice several hours following intranasal infection with HSV-1 (Table 1). Replication of virus in the lungs of treated mice was reduced by one $\log_{10}$ at this dosage. Some protection was also observed when $5 \times 10^6$ U/kg was administered shortly after virus infection, but this dose had no significant effect on pulmonary virus replication. Lower doses were not effective when administered as a single bolus immediately following infection. rIFN-αB/D was also effective in a more aggressive infection caused by the MacIntyre strain of HSV-1. As demonstrated in Table 2, two to five consecutive daily injections given by various routes and starting approximately 1 h after infection enhanced both survival rates and mean survival time of those animals that eventually succumbed to infection. Significant protection (8 out of 10 mice; $p \leq 0.01$) was observed following two consecutive i.p. or i.v. inoculations with IFN on the day of infection and 1 day later.

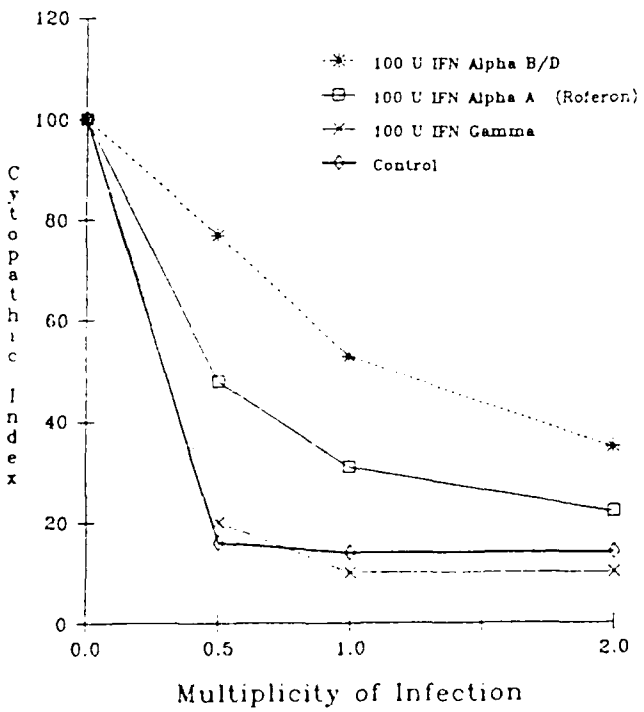


**FIG. 1.** Inhibition of HSV-1 cytopathic effects in cultured human monocytes. Freshly isolated monocytes were incubated for 10 days prior to an 18-h preincubation with IFN and infection with the VR3 strain of HSV-1. Virus-induced cytopathology was quantitated using the uptake of neutral red dye as a measure of viability 48 h post infection. The values shown represent the mean of three wells.

TABLE 1. EFFECT OF rHuIFN-αB/D ON HSV-1/VR3 INFECTION

| *Treatment*[a] | *Survivors* | *MST*[b] *(days)* | *Lung virus*[c] *(pfu/g)* |
|---|---|---|---|
| PBS | 3/25 | 8.7 | $2.7 \times 10^{7}$ |
| rHuIFN-αB/D ($5 \times 10^{6}$ U/kg) | 11/25* | 10.0 | $1.4 \times 10^{6}$ |
| rHuIFN-αB/D ($5 \times 10^{7}$ U/kg) | 21/25** | 9.8 | $2.7 \times 10^{[illegible]}$** |

[a]PBS or rHuIFN-αB/D was administered i.v. (0.2 ml) 3 h after intranasal instillation (0.05 ml) of HSV-1/VR3 strain ($4 \times 10^{4}$ pfu).
[b]Mean survival time of animals which died within a 21-day observation period.
[c]Plaque forming units in 10% lung homogenates. Data reflect values from 3 mice in each group.
**$p < 0.001$ when compared to control values.
*$p \leq 0.01$.

TABLE 2. EFFECT OF rHuIFN-αB/D ON HSV-1/MCINTYRE INFECTION

| *Treatment (U/kg • day)* | *Route* | *Number of injections* | *Day of treatment relative to infection*[a] | *Survivors* | *MST*[b] *(days)* |
|---|---|---|---|---|---|
| | | | *Experiment 1* | | |
| None | | | | 0/10 | 7.5 |
| PBS | i.v. | 5 | 0,1,2,3,4 | 1/10 | 8.4 |
| rHuIFN-αB/D | | | | | |
| $5 \times 10^{5}$ | i.v. | 5 | 0,1,2,3,4 | 5/10* | 8.2 |
| $5 \times 10^{6}$ | i.v. | 5 | 0,1,2,3,4 | 2/10 | 9.1 |
| $5 \times 10^{7}$ | i.v. | 5 | 0,1,2,3,4 | 7/10* | 10.7 |
| | | | *Experiment 2* | | |
| PBS | i.p. | 2 | 0,1 | 0/10 | 8.4 |
| rHuIFN-αB/D | | | | | |
| $5 \times 10^{7}$ | i.p. | 2 | 0,1 | 8/10* | 8.5 |
| $5 \times 10^{7}$ | i.v. | 2 | 0,1 | 8/10* | 10.0 |
| $5 \times 10^{7}$ | s.c. | 2 | 0,1 | 5/10* | 9.2* |

[a]Mice were infected i.p. with 3,000 pfu (1st exp.) and 4,000 pfu (2nd exp.).
[b]Mean survival time of animals which died within a 21-day observation period.
*$p \leq 0.01$.

### *Therapeutic activity of rHuIFN-αB/D in mice infected with Rauscher or Friend leukemia virus*

The effect of rHuIFN-αB/D on RMLV infection is illustrated in Fig. 2. Note that a transient effect was observed for at least 10 days following the last treatment day (day 20). No augmentation in survival rates were observed following IFN treatment; however, a prolongation in mean survival times was evident. More impressive, however, was the effect of rHuIFN-αB/D on FMLV infection (Fig. 3). A significant enhancement of mean of survival time and number of long-term survivors

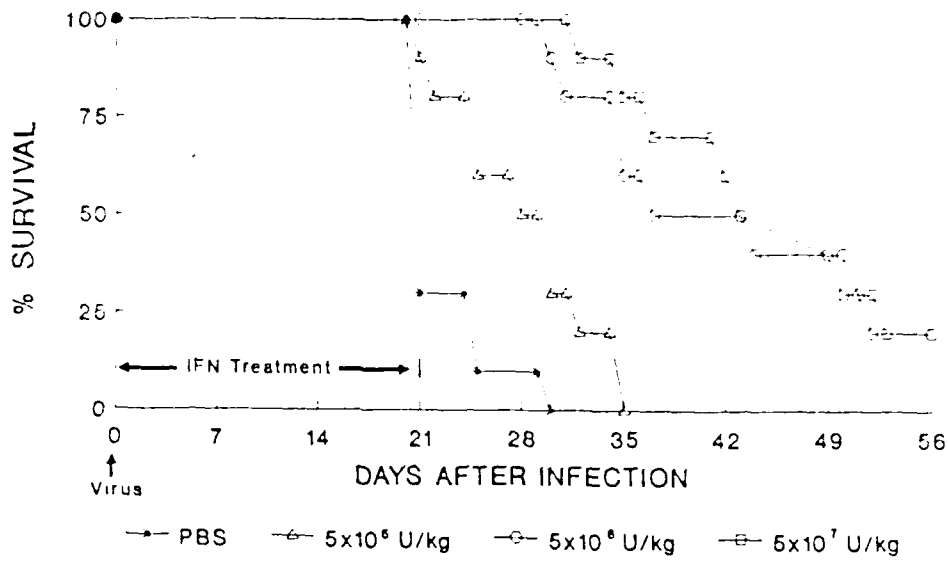


**FIG. 2.** Therapeutic activity of rHuIFN-αB/D in RMLV infection. Female BALB/C mice infected i.p. with 10 $LD_{50}$ of virus and given the indicated doses of interferon (s.c.) every second day for 21 days beginning on the day of the virus infection. $n = 20$ mice per group. $p \leq 0.01$ for MST of mice receiving $5 \times 10^6$ or $5 \times 10^7$ U/kg of rHuIFN-αB/D.

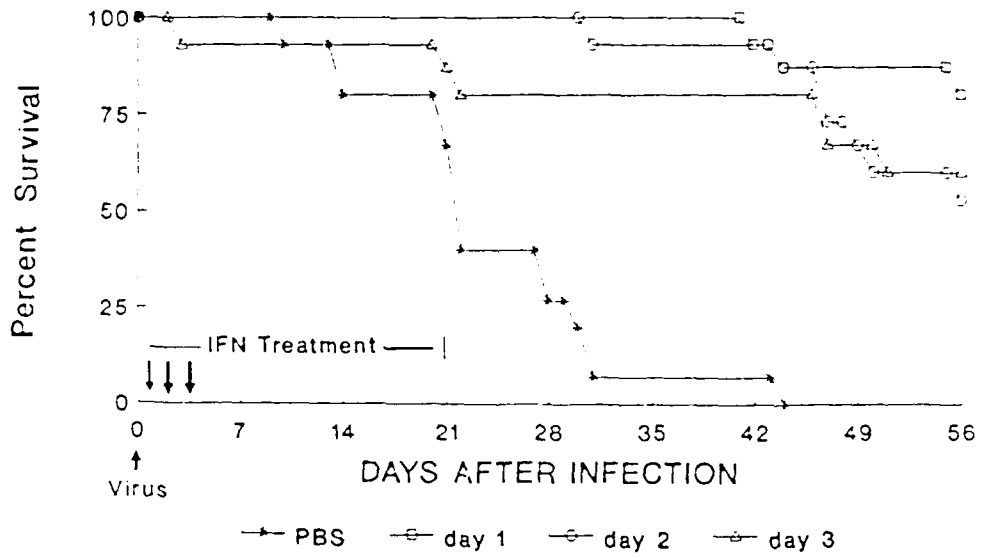


**FIG. 3.** Therapeutic activity of rHuIFN-αB/D ($5 \times 10^7$ U/kg) in FMLV infection. Female DBA/2 mice were infected i.p. with 10 $LD_{50}$ of virus and given IFN (s.c.) every second day for 21 days beginning on day 1, 2, or 3 following infection. $n = 20$ mice per group.

was observed when IFN treatment ($5 \times 10^7$ U/kg) was initiated several days after virus infection. Lower dosages ($5 \times 10^6$ U/kg) prolonged mean survival time but did not enhance long-term survival (data not shown).

Spleens from mice receiving rHuIFN-αB/D following RMLV or FMLV infection were significantly smaller than those from untreated mice and the number of circulating white blood cells was reduced as was the level of reverse transcriptase in spleens of infected mice (Table 3).

TABLE 3. EFFECT OF rHuIFN-αB/D ON RMLV AND FMLV INFECTIONS

| | Treatment[a] | Spleen weight (mg)[b] Mean ± SD | WBC[b] Mean ± SD (× 10⁶) | Reverse transcriptase (cpm)[b] |
|---|---|---|---|---|
| RMLV | | | | |
| | PBS | 2,852 ± 583 | 166.4 ± 36.5 | 7,928 |
| | 5 × 10⁵ U/kg | 2,046 ± 339 | 103.4 ± 23.5 | 4,373 |
| | 5 × 10⁶ U/kg | 1,641 ± 318* | 114.3 ± 23.9 | 3,04[illegible] |
| | 5 × 10⁷ U/kg | 1,110 ± 587* | 79.4 ± 25.7 | 5,33[illegible] |
| FMLV | | | | |
| | Untreated | 1,36[illegible] ± 346 | 226.4 ± 4.9 | 5,825 |
| | 5 × 10⁷ U/kg Day 1 | 389 ± 128* | 66.7 ± 22.1 | 2,506 |
| | 5 × 10⁷ U/kg Day 2 | 408 ± 159* | 48.5 ± 18.8 | 2,306 |
| | 5 × 10⁷ U/kg Day 3 | 420 ± 142* | 59.8 ± 27.2* | 3,448 |

[a]IFN treatment (s.c.) was initiated on the day of virus infection and continued every second day for 21 days.
[b]Spleen weight, WBC, and reverse transcriptase levels were examined 15 days post infection. Reverse transcriptase levels in 10% splenic homogenates. Five mice per treatment group were examined.
*$p < 0.05$.

### *Activity of rHuIFN-αB/D in a murine model of retroviral-induced immunodeficiency*

Infection of C57BL/6 mice with LP-BM5 virus results in varying degrees of immunosuppression which intensifies as the time post infection increases. We have monitored immune changes in mice at both early (2–3 weeks) and late (2–3 months) times following infection to assess the effects of IFN or AZT treatment. Administration of rHuIFN-αB/D every second day for 14 days following infection partially restored concanavalin A responsiveness, suppressed splenomegaly, and delayed the appearance of lymphadenopathy during the late stages of disease (Table 4). A more pronounced but similar therapeutic effect was also observed when AZT (0.2 mg/ml) was administered in the drinking water.

Moreover, the therapeutic activity of both drugs appeared to increase when lower AZT concentrations (0.02 mg/ml) were used together with IFN-α throughout a 21-day treatment schedule (Table 5). Non-immunosuppressed adult C57BL/6 mice were fully resistant to intranasal infection with HSV-1/VR3 while mice receiving LP-MB5 virus 60 days prior to HSV-1 infection were susceptible. Treatment with either AZT or rHuIFN-αB/D alone had little effect on the ability of LP-BM5 infected mice to resist HSV-1 infection. In contrast, note that in addition to the restoration of mitogen responsiveness and suppression of spienomegaly, 75% of the mice receiving combination therapy were resistant to HSV-1 superinfection.

## DISCUSSION

Recombinant human lymphoblastoid IFNs-αB and -αD can be distinguished from one another based on their amino acid sequence (35 amino acid difference) and specific antiviral and antiproliferative activities.[2] When assayed on human cells, IFN-αB is about 30 times more active than IFN-αD; however, when assayed on mouse cells, IFN-αB is 10 times less active than IFN-αD.[15,16] Since

TABLE 4. THERAPEUTIC ACTIVITY OF rHuIFN-αB/D ADMINISTERED EVERY OTHER DAY FOR 14 DAYS FOLLOWING LP-BM5 INFECTION

| | *Indicators of disease progression*[a] | | | | |
|---|---|---|---|---|---|
| | | | *Lymphadenopathy*[d] | | |
| *Treatment*[b] | *ConA response*[c] *(absorbance ± SD)* | *Spleen weight (mg) Mean ± SD* | *Day 60* | *Day 90* | *Day 105* |
| Uninfected | (−) 0.639 ± 0.021<br>(+) 1.614 ± 0.098 | 69 ± 13 | 0/6 | 0/6 | 0/6 |
| PBS<br>virus infected | (−) 0.637 ± 0.017<br>(+) 0.931 ± 0.016 | 153 ± 18 | 6/6 | 6/6 | 6/6 |
| AZT<br>(0.2 mg/kg) | (−) 0.563 ± 0.006<br>(+) 1.231 ± 0.079 | 70 ± 13 | 0/6 | 0/6 | 2/6 |
| rHuIFN-αB/D<br>($5 \times 10^7$ U/kg) | (−) 0.573 ± 0.016<br>(+) 1.047 ± 0.018 | 112 ± 6 | 0/5 | 0/5 | 4/5 |

[a]All determinations except for lymphadenopathy were made 21 days post infection. Five mice per treatment group were evaluated.

[b]Mice were infected i.p. with 0.5 ml of undilute virus and treatment initiated several hours later. AZT was administered in drinking water (each mouse consumed an average of 3 ml/day) and rHuIFN-αB/D was administered s.c. every second day beginning on the day of infection and continued for 14 days. Control mice received PBS on treatment days.

[c]Proliferative response of splenocytes to concanavalin A; (−) no ConA in cultures; (+) ConA added. See Materials and Methods for details of colorimetric assay.

[d]Appearance of enlarged thoracic (parathymic), axial, and inguinal lymph nodes.

TABLE 5. THERAPEUTIC ACTIVITY OF rHuIFN-αB/D ADMINISTERED EVERY OTHER DAY FOR 21 DAYS FOLLOWING LP-BM5 INFECTION

| | *Indicators of disease progression*[a] | | |
|---|---|---|---|
| *Treatment*[b] *group* | *ConA response*[c] *(absorbance ± SD)* | *Spleen weight (mg) Mean ± SD* | *HSV-1 superinfection*[d] *(% survival)* |
| None<br>Uninfected | (−) 0.571 ± 0.019<br>(+) 1.686 ± 0.090 | 71 ± 15 | 100 |
| PBS<br>LP-BM5 infected | (−) 0.523 ± 0.020<br>(+) 0.928 ± 0.030 | 167 ± 33 | 25 |
| AZT (0.02 mg/ml) | (−) 0.614 ± 0.015<br>(+) 1.463 ± 0.013 | 143 ± 39 | 38 |
| rHuIFN-αB/D ($5 \times 10^7$ U/kg) | (−) 0.638 ± 0.021<br>(+) 1.264 ± 0.021 | 147 ± 27 | 29 |
| rHuIFN-αB/D ($5 \times 10^7$ U/kg)<br>+ AZT (0.2 mg/ml) | (−) 0.607 ± 0.019<br>(+) 1.425 ± 0.033 | 82 ± 12 | 75 |

[a]ConA response and spleen weight taken 21 days post infection. Five mice per treatment group were examined.

[b]Mice were infected i.p. with 0.5 ml of undilute virus and therapy initiated several hours later. AZT was administered in drinking water (each mouse consumed an average of 3 ml/day). rHuIFN-αB/D was given s.c. beginning on day of infection and continued for 21 days. Control mice received PBS on treatment days.

[c]Proliferative response of splenocytes to concanavalin A. (−) No ConA in cultures; (+) ConA added.

[d]HSV-1/VR3 superinfection (intranasal challenge with $4 \times 10^5$ pfu) was done 60 days after infection with LP-BM5. Ten mice per treatment group were infected.

the mechanism controlling species specificity appears to depend on receptor affinity, it has been proposed that the rHuIFN-αB/D hybrid probably adopts a structural conformation which is compatible with the receptors of a variety of animal species.[3] This unique structural feature has enabled us to examine this hybrid molecule in both human monocytes and mice.

When examined in cultured human monocytes, rHuIFN-αB/D was superior to rHuIFN-γ and -αA in preventing cell destruction and in inhibiting virus replication. Similarly, IFN-γ has been reported to lack the ability to induce an antiviral state in human peripheral blood lymphocytes infected with HIV.[29] Why such differences exist is unclear, but the reason may reside in the affinity of each IFN species for the monocyte or lymphocyte receptor, or in the ability of each to induce specific intracellular antiviral proteins.[17] Future studies will examine the proteins induced by rHuIFN-αB/D as well as IFN-αA and IFN-γ in cultured human monocytes and lymphocytes.

We have shown that a single dose of rHuIFN-αB/D ($5 \times 10^7$ U/kg) on the day of infection was able to augment resistance to HSV-1/VR3 induced pneumonitis. An inhibition of pulmonary virus replication was also observed at this dosage. In addition, when rHuIFN-αB/D was administered shortly after infection of mice with an aggressive strain of HSV-1/McIntyre and again on days 1–4 post infection, resistance to infection was greatly augmented. Interestingly, treatment beyond the first day post infection was of little value, indicating that rHuIFN-αB/D acted at an early stage in viral replication. Similarly, resistance to HSV-1 infection in newborn and adult mice has been correlated with the rapid appearance of IFN.[27] While it is quite likely that direct IFN action at the level of viral RNA or protein synthesis was responsible for some of these effects, augmentation of the antiviral activity of effector cells such as natural killer cells and macrophages may have resulted in their participation as well.[27,28]

It has been proposed that IFN inhibits the replication of retroviruses by altering the cell membrane structure and/or function that prevents the insertion of viral core precursor molecules into the cell membrane.[18–20] In our study, we were able to show a transient effect of rHuIFN-αB/D on three retroviral infections. Of these, Friend virus-infected mice had the best therapeutic response with respect to prolongation of mean survival time and number of long-term survivors. In addition, treatment initiated 3 days post infection was as good or better as that initiated on the day of infection. When rHuIFN-αB/D was given shortly after infection, there was a dramatic antiviral effect as indicated by reverse transcriptase levels, white blood cell counts and suppression of splenomegaly. In contrast, treatment started at late times during infection (*i.e.*, 15 days post infection) appeared to exert more of an antileukemic activity (data not shown). Similar antiviral and antiproliferative activities have been reported for chemotherapeutic agents in both the Rauscher[21] and Friend[8] virus models. Why rHuIFN-αB/D appeared to be more active in mice infected with FMLV is not clear and is currently under investigation.

In the murine model of AIDS previously described by Mosier *et al.*,[9,12,13] rHuIFN-αB/D was again shown to have a transient effect on the development of virus-induced immunosuppression and lymphadenopathy. As is the case for HSV-1, RMLV, and FMLV infections, the observed effect on LP-BM5 infection was probably due to a reduction in viral replication (which may have decreased the degree of virus-induced immunosuppression) as well as to the augmentation of the immune system. Nonetheless, the antiproliferative effects of IFN-α, in particular inhibition of proliferating B-lymphocytes, may also play a role.[22]

Synergism between azidothymidine and rHuIFN-αA for the *in vitro* inhibition of HIV has been reported.[23] In addition, Connell *et al.*[24] reported a synergistic effect when rHuIFN-αA/D and acyclovir were used in a murine model of lethal HSV-1 infection. In our study, we have shown an enhancement of the antiviral activities of AZT and rHuIFN-αB/D when both are given to mice infected with the immunosuppressive LP-BM5 virus. Whether or not this finding represents an additive or synergistic effect remains to be determined. Nonetheless, the combination of both drugs, each working at different levels in the inhibition of virus replication, may be of relevance in the treatment of human retroviral infections.

The data presented in this study clearly reveal the *in vivo* antiviral activity of rHuIFN-αB/D in murine models of retroviral and herpetic disease. In light of the encouraging results using rHuIFN-αB/D in combination with AZT, future studies will explore potentiating effects of this hybrid IFN when used together with other virustatic and immunomodulating agents.

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